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PROGRESS IN BRAIN RESEARCH

Advances in Brain Vasopressin

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Preface

This Volume on Brain Vasopressin was conceived about two years ago by the Editors who themselves are already engaged in studies on the central nervous system effects of the neurohypophysial hormones for many years. Since the discovery of vasopressin and oxytocin as peripheral hormones, these peptides have fulfilled a role as 'prototype'. These hormones were the only ones known in the 1950s to be produced by the brain. Their main endocrine functions in the periphery were already well described. The first observation which suggested a central nervous system effect of vasopressin came from studies on avoidance behavior in the early 1960s. These led to the hypothesis that vasopressin and related peptides were involved in learning and memory processes. Further studies on these principles in many laboratories around the world revealed that they possessed an array of effects on such brain functions as learning and memory, rewarded behavior and drug tolerance, social, reproductive and feeding behavior, temperature and cardiovascular regulation. We now know that many other neuropeptides display this diversity in actions and functions.

In thirty years we have come to know much more about the central nervous system effects and functions of vasopressin. Most of that information has been reviewed extensively in various periodicals. Would there be enough new information to guarantee a book on this topic? We were convinced that we could still learn from the principles provided by vasopressin research and invited those who are actively engaged in studies on the central nervous system effects of vasopressin to contribute a chapter. Many experienced authors in this field whom we invited contributed important chapters to this volume. We also chose to include young authors to provide their recent data and views. The volume comprises animal studies on the neuroanatomy of the brain vasopressin system, on electrophysiological properties and cell biology of vasopressin neurons, on the molecular and electrophysiological actions of and responses to vasopressin on the brain, and on the influence of vasopressin on autonomic functions. A large section is devoted to the behavioral effects of vasopressin and it ends with studies on the brain vasopressin system in humans. We allowed overlap in different contributions on related topics in order to make the reader aware of the different interpretations and hypothesis on the current issues, as for example the role and mode of action of vasopressin metabolites.

The endeavor appeared to be very worthwhile since a lot of new data have been amassed in recent years. This makes this volume a highly relevant and useful book of reference in particular for those who are working on this 'first' neuropeptide, but also for (young) investigators who consider entering the field of neuropeptides, and as a book of reference for teachers and students. In this Volume it is noticeable that vasopressin is still serving as a prototype for new areas of neuropeptide research. In addition it shows how important a multidisciplinary approach is to unravel the function of a neuropeptide in the brain.

I.J.A. Urban, J.P.H. Burbach and D. De Wied

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Contents

SECTION 1

Neuroanatomy of the brain vasopressin systems

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
Progress in Brain Research, Vol. 119

CHAPTER 1.1

Anatomy and function of extrahypothalamic vasopressin systems in the brain

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The most prominent sites of vasopressin (VP) production in the rat brain are the paraventricular nucleus, the supraoptic nucleus, the suprachiasmatic nucleus, the bed nucleus of the stria terminalis (BST), and the medial amygdaloid nucleus (MA). Recently a number of new sites have been suggested, including the hippocampus, the diagonal band of Broca, and the choroid plexus. This chapter shows how differential regulation of these VP systems can be exploited to identify the contributions of individual VP systems to the various central functions in which VP has been implicated. It will focus on the development, anatomy, and function of the sexually dimorphic VP projections of the BST and MA. This system contains more cells and has denser projections in males than in females. This system is also extremely responsive to gonadal steroids as it only produces VP in the presence of gonadal steroids. It has been implicated in sexually dimorphic functions such as aggressive behavior as well as in non-sexually dimorphic functions such as social recognition memory. Using comparative studies done in prairie voles as an example, this chapter makes the case that the VP projections of the BST and MA may simultaneously generate sex differences in some brain functions and behaviors and prevent them in others.

Anatomy of vasopressin pathways in the brain

Traditionally, physiological effects of vasopressin (VP) have been reported before the systems underlying these effects were described. The vasopressor effects of VP (Oliver and Schäfer, 1895) were known long before Bargmann and Scharrer (1951) had proposed that the neurons projecting from the supraoptic (SON) and paraventricular nucleus (PVN) to the posterior pituitary were the source of this hormone. Similarly, effects of VP on learning and memory (De Wied, 1965) were reported before the central projections of VP neurons were found. Presently, powerful anatomical techniques such as immunocytochemistry, in situ hybridization, neuroanatomical tract tracing, and ligand binding assays have revealed several different systems in the brain that either produce or respond to VP. Meanwhile, the list of possible functions that may be influenced by central VP continues to grow and includes, in addition to learning and memory, such functions as thermoregulation, cardiovascular functions, social recognition memory, sexual behavior, and aggressive behavior (De Wied et al., 1993; Engelmann et al., 1996). It is far from clear, however, specifically which VP-expressing system is responsible for the various VP effects. This chapter addresses first the anatomy of VP systems in the rat brain. Then, we illustrate how differences in the regulation of these systems can be exploited to study individual contributions of the different VP systems to brain function, using the sexually dimorphic projections of the bed nucleus of the stria terminalis (BST) and medial amygdaloid nucleus (MA) of the rat as an example.

The VP projections in the brain have been extensively traced in rats, mostly by immunocytochem-

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istry, in situ hybridization, lesion studies, and neuroanatomical tract-tracing. VP is synthesized in several cell groups, each of which projects to distinct areas in the brain (Fig. 1). Magnocellular neurons of the paraventricular (PVN) and supraoptic nuclei (SON) project to the median eminence and posterior pituitary, where they release VP as a hormone that influences, for example, water retention and ACTH release. Parvocellular neurons of the PVN project to the hindbrain and spinal cord, probably to control autonomic functions such as heart rate and blood pressure (Sawchenko and Swanson, 1982). The suprachiasmatic nucleus projects to areas around the third ventricle, probably regulating circadian functions (Hoorneman and Buijs, 1982). In addition, there are several cell groups that were discovered only after animals were pretreated with the axonal transport blocker colchicine. The most prominent of these cell groups are found in the intermediate and medial subdivisions of the BST, and in the anterior and posterodorsal divisions of the MA (Caffé and Van Leeuwen, 1983; Van Leeuwen and Caffe, 1983). The VP neurons in these extrahypothalamic areas project to forebrain structures, such as the lateral septum, the lateral habenular nucleus, and to midbrain structures such as midbrain central gray, dorsal raphe nucleus, pontine peripeduncular nucleus and the locus coeruleus (De Vries and Buijs, 1983; Caffé et al., 1987). The VP-expressing neurons of the BST and MA share a similar morphology (Fig. 2) and show similar patterns of responsiveness to gonadal steroids. Although the VP projections of the BST and MA may each make their own unique contributions to brain function, for the sake of simplicity, we discuss these neurons as one functional system. Other groups of VP-immunoreactive neurons that are found after colchicine treatment are in the dorsomedial hypothalamic nucleus and the locus coeruleus. The locus coeruleus does not appear to be a significant source of VP innervation in the brain. No VP mRNA is found in this nucleus (Urban et al., 1990) whereas it is found in other areas where colchicine

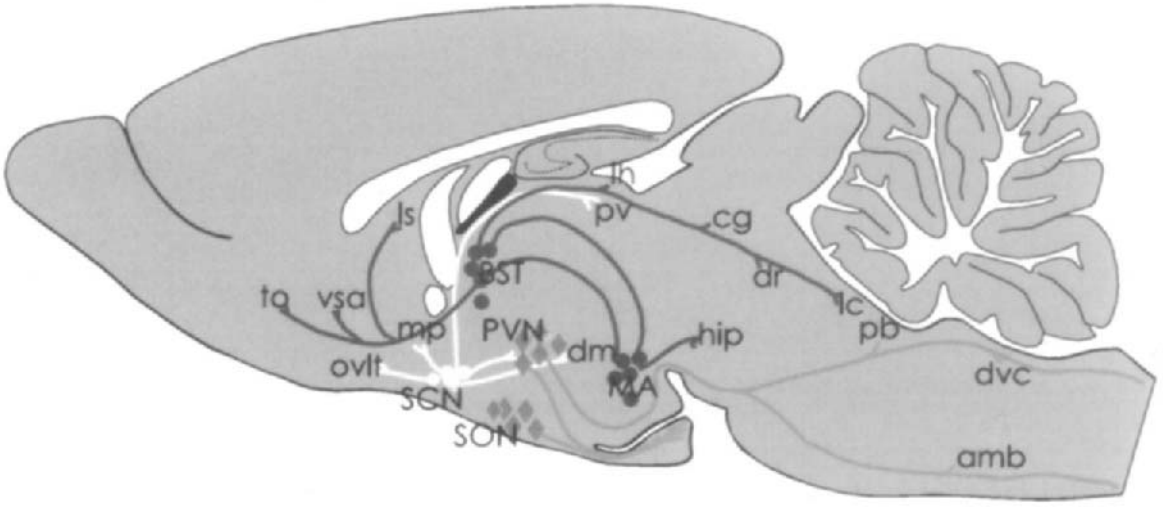


Fig. 1. Scheme of the most prominent VP-immunoreactive projections; dark grey circles: from the bed nucleus of the stria terminalis (BST) and medial amygdaloid nucleus (MA) to the lateral septum (ls), ventral septal area (vsa), olfactory tubercle (to), lateral septum (ls), lateral habenular nucleus (lh), midbrain central grey (cg), dorsal raphe nucleus (dr), locus coeruleus (lc), and ventral hippocampus (hip); off-white circles: from the suprachiasmatic nucleus (SCN) to the organum vasculosum laminae terminalis (ovlt), medial preoptic areas (mp), periventricular (pv) and dorsomedial nucleus of the hypothalamus (dm); light grey diamonds: from the paraventricular nucleus (PVN) to the parabrachial nucleus (pb), dorsal vagal complex (dvc), and ambiguus nucleus (amb), plus projections from PVN and supraoptic nucleus (SON) to the neurohypophysis (adapted from De Vries et al., 1985).

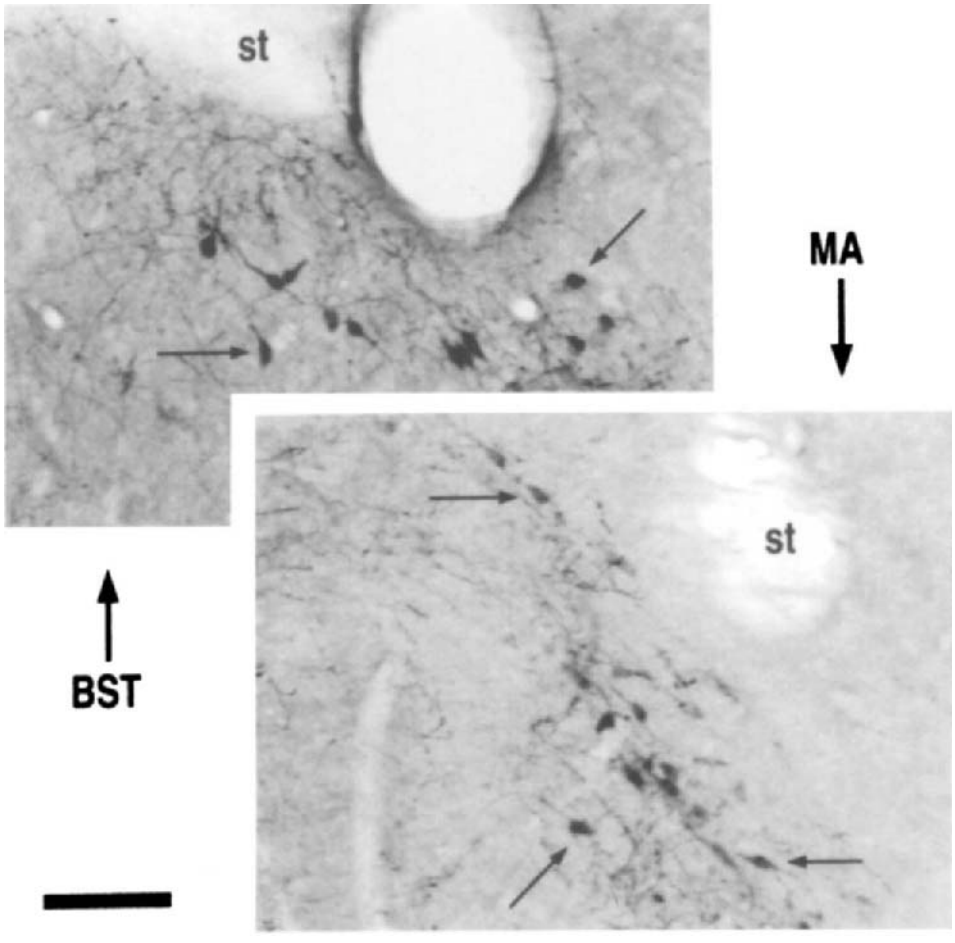
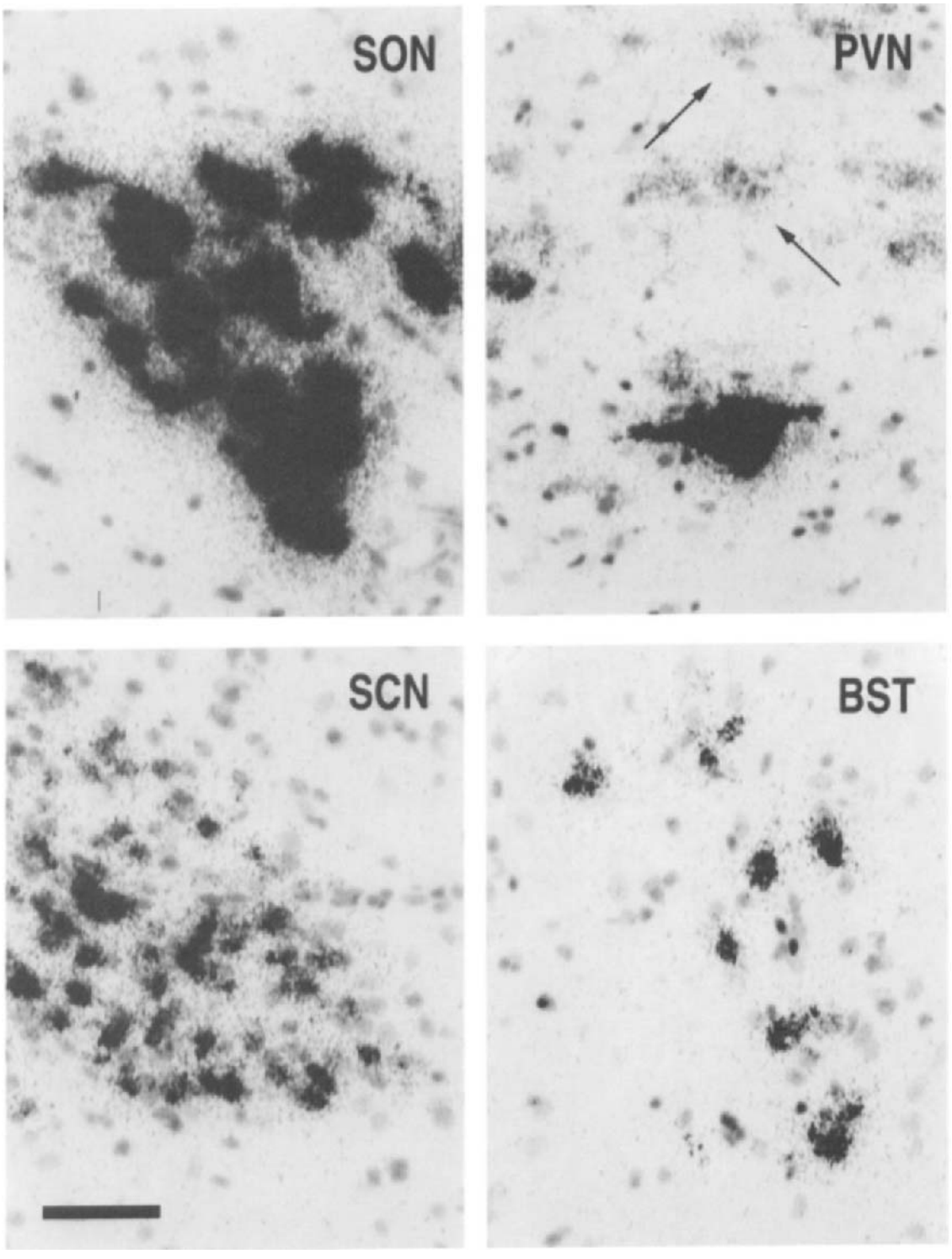


Fig. 2. VP-immunoreactive cells (arrows) in the bed nucleus of the stria terminalis (BST) and medial amygdaloid nucleus (MA) in a male rat photographed in the same section cut in a plane intermediate to the transverse and horizontal plane; st, commissural part of the stria terminalis (bar = 50 μm).

boosts VP immunostaining (Miller et al., 1988; Urban et al., 1990). In addition, locus coeruleus lesions do not reduce VP immunoreactivity elsewhere in the brain (Caffé et al., 1988). However, the presence of VP-immunoreactive cells in the locus coeruleus after colchicine treatment suggests that this nucleus has the capacity to synthesize VP under some conditions.

It is questionable whether immunocytochemistry gives a complete picture of all possible sites of VP synthesis and release. Push-pull experiments have measured VP in the cerebrospinal fluid in such areas as the dorsal hippocampus and the striatum where usually no fibers are observed (Landgraf et al., 1991). In addition, although the distribution of VP receptors often closely matches the distribution of VP fibers, receptors are also found in areas such as the dorsal hippocampus, where vasopressin-immunoreactive fibers are scarce or absent (Johnson et al., 1993; Szot et al., 1994). Highly sensitive in situ hybridization assays have recently suggested a number of additional areas which may be sources of VP innervation. The horizontal limb of the diagonal band of Broca contains a low number of cells that label for VP mRNA (Planas et al., 1995a). Even more recently, VP mRNA was found in


SON
PVN
SCN
BST

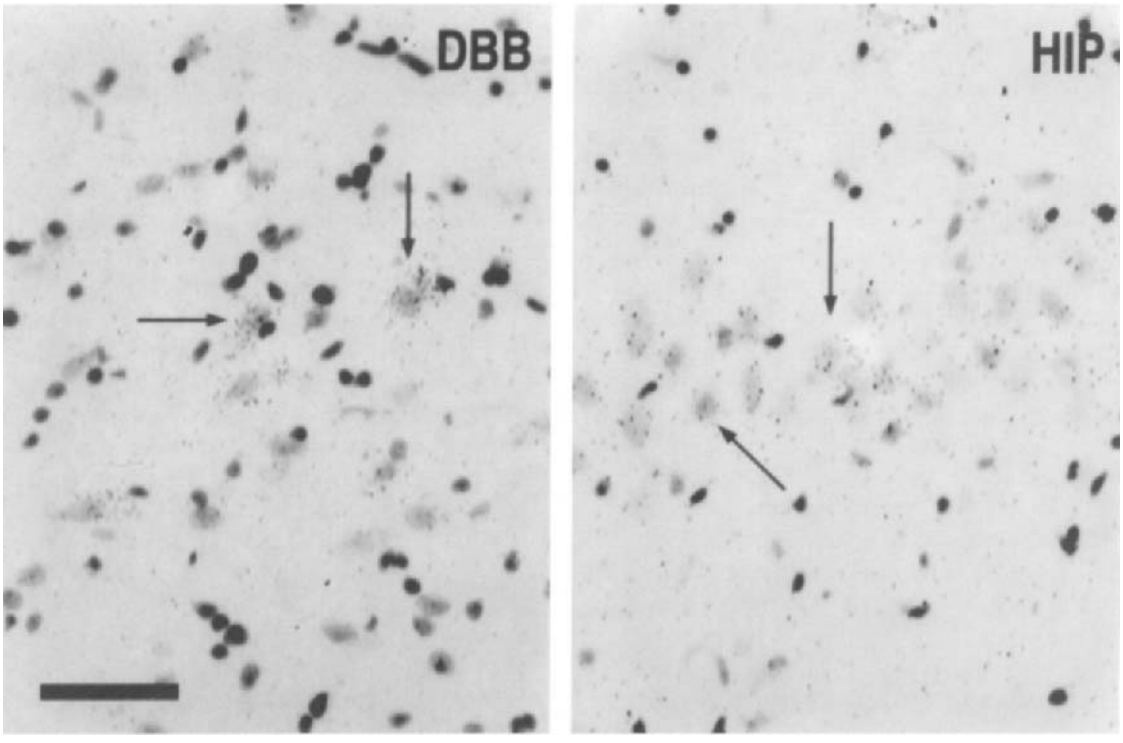


Fig. 4. VP messenger RNA labeling (black silver grains over individual neurons) photographed in sections of the same male rat at the same magnification (bar = 25 μm). Grey objects are the nuclei of individual neurons and glia cells counterstained with cresyl violet. DBB, diagonal band of Broca; HIP, CA1 of the hippocampus. Arrows point at individual neurons.

cells in the dorsal tegmental area, the parabrachial nucleus, and in the CA1 and CA3 fields in the hippocampus and the dentate gyrus (Hallbeck et al., 1999). The choroid plexus may be another possible source of central VP, because it also contains cells that can be labeled for VP mRNA (Chodobski et al., 1997). VP secreted by these cells could reach many potential targets in the brain. Although the levels of VP mRNA in these areas are much lower than the levels in the suprachiasmatic nucleus and the bed nucleus of the stria terminalis, the large number of cells involved, especially including those found in the hippocampal complex, could make it a considerable source of VP in the brain. However, the presence of VP mRNA in these areas remains to be confirmed using other methods. It is also unclear whether the peptide VP is synthesized and released by these cells, not to mention the uncertainties about the physiological role of VP secreted by these cells. Finally, VP mRNA has been detected in the median eminence and neurophypophysis where it may be present in axons of magnocellular neurons of the SON and PVN (Trembleau et al., 1995).

Fig. 3. VP messenger RNA labeling (black silver grains over individual neurons) photographed in sections of the same male rat at the same magnification (bar = 25 μm). Grey objects are the nuclei of individual neurons and glia cells counterstained with cresyl violet. SON, supraoptic nucleus showing magnocellular neurons; PVN, paraventricular nucleus showing one magnocellular neurons plus several parvocellular neurons, which presumably project to the hindbrain and spinal cord; SCN, suprachiasmatic nucleus. BST, bed nucleus of the stria terminalis.

Steroid hormone effects on VP pathways in the brain

Different neuronal groups regulate VP expression differently from each other. This can be inferred from the different levels of VP messenger RNA in each of these areas (Figs. 3 and 4). It is also evident from the effects of steroid hormones on VP expression. For example, adrenal steroids block VP mRNA expression in the parvocellular neurons of the PVN that project to the median eminence, but these steroids do not significantly affect other VP neurons in the brain (Sawchenko, 1987; Urban et al., 1991). Conversely, gonadal steroids must be present in order for BST and MA neurons to express VP (De Vries et al., 1984, 1985; Van Leeuwen et al., 1985; Miller et al., 1989a). Although castration causes most VP mRNA to disappear from BST and MA cells within a week (Miller and De Vries, 1992), it takes 2–3 months before all VP immunoreactivity disappears from BST and MA projections (Fig. 5) (De Vries et al., 1984; Mayes et al., 1988). Although gonadal hormones influence the production of VP in the PVN and SON (O'Keefe et al., 1995), they do not have noticeable effects on the central projections from the PVN (De Vries et al., 1985), nor do they significantly affect VP production in the SCN (De Vries et al., 1984, 1985). However, changes in gonadal hormone levels dramatically influence central VP neurotransmission, because BST and MA neurons provide the lion's share of the VP innervation of the brain.

The discrepancy between the rate of decline of peptide levels and VP mRNA in the BST and MA after gonadectomy suggests that gonadectomy rapidly blocks VP release and keeps the remaining VP stored in terminals for months. There is indirect evidence that VP release from BST and MA terminals is indeed blocked in castrated rats. Intracerebroventricular injections of VP cause motor disturbances in rats, but only if these rats have been primed with a similar injection of VP 2 days earlier (Poulin and Pittman, 1991). Peripheral injections of hypertonic saline prime rats equally well for the motor effects of VP as do intracerebroventricular injections of VP (Poulin and Pittman, 1991), presumably because hypertonic saline stimulates the release of VP from BST and MA projections (Demotes-Mainard et al., 1986; Landgraf et al., 1988). In castrated rats, however, intracerebroventricular injections can still prime rats for the motor effects of VP but peripheral injections of hypertonic saline cannot prime rats anymore, presumably because castration has blocked the release of endogenous VP in the septal area (Poulin and Pittman, 1991). In rats, this presumed gonadectomy-induced reduction in VP release does not seem to influence receptor density or sensitivity as do changes in other neurotransmitter systems (Catt et al., 1979). Castration does not change the distribution of VP binding sites, the number and affinity of VP receptors, and VP-stimulated phosphotidyl-inositol hydrolysis in septal tissue, nor does it affect the ability of VP to prime septal tissue for the motor effects of subsequent VP injections (Tribollet et al., 1988; Poulin and Pittman, 1991).

Gonadal hormones influence VP expression in the BST and MA by estrogenic as well as androgenic mechanisms. Estradiol, which in addition to being an ovarian hormone is a metabolite of testosterone (Naftolin et al., 1975), partially restores VP immunostaining in castrated male rats, while 5α-dihydrotestosterone (DHT), an androgenic metabolite of testosterone (Lieberburg and McEwen, 1975), does not restore VP immunostaining by itself. However, DHT enhances the effects of estradiol on VP immunostaining (De Vries et al., 1985). The same synergistic interaction of estradiol and DHT is found for VP mRNA expression (Fig. 6) (De Vries et al., 1984). Androgens and estrogens may influence VP production by directly acting on VP-immunoreactive cells, because virtually all VP-immunoreactive cells in the BST and MA in males are immunoreactive for estrogen as well as androgen receptors (Fig. 7) (Axelson and Van Leeuwen, 1990; Zhou et al., 1994).

Recently, we have tested whether local actions of estradiol are indeed crucial for the effects of gonadal hormones on VP expression by implanting male rats bilaterally with cannulas aimed at the BST. One cannula was filled with the pure anti-estrogen ICI 182,780, while the other cannula was filled with cholesterol or kept empty. The number of VP-immunoreactive cells in the BST on the ICI

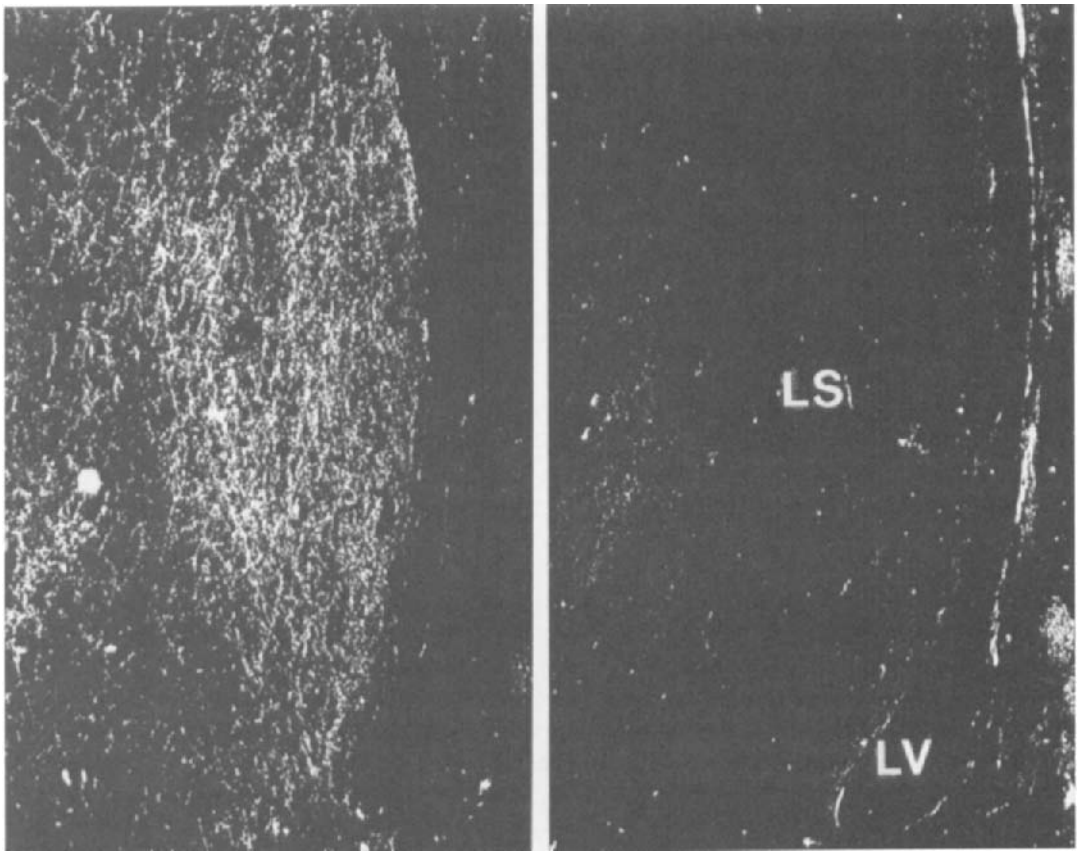


Fig. 5. Dark field-illuminated section showing VP-immunoreactive fibers in the lateral septum (LS) of an intact (left) and castrated male rat (right). Note that after castration some non-branching, thick-calibre fibers remain. LV, ventral tip of lateral ventricle.

182,780 side was significantly lower than on the cholesterol or blank side, suggesting indeed that gonadal hormones stimulates VP expression by activating local estrogen receptors (Chung et al., 1996). Because pure antiestrogens presumably prevent estrogen-induced gene expression by increasing estrogen receptor turnover and by blocking estrogen receptor binding to response elements in the DNA (Fawell et al., 1990; Dauvois et al., 1992), the reduction of VP expression by ICI 182,780 suggests that gonadal steroids stimulate VP expression by activating estrogen receptors that alter genomic expression.

It is unknown whether estrogen and androgen receptors directly target the VP gene or whether they influence the expression of other gene products that, in turn, influence VP gene expression. There are no clearly recognizable androgen- and estrogen-responsive elements on the promoter region of the VP gene (Adan and Burbach, 1992; Young, 1992). It is even questionable whether androgens and estrogens influence VP mRNA transcription at all because nuclear run-on assays of BST tissue failed to show an effect of castration on the rate VP gene transcription. Instead, gonadal hormones may regulate VP mRNA levels at a posttranscriptional level because castration decreased the length of the poly (A) tail of VP mRNA in the BST while testosterone increased it, thereby presumably influencing the stability of VP mRNA (Carter and Murphy, 1993).

It is also unknown how androgens and estrogens influence each other's effectiveness in stimulating

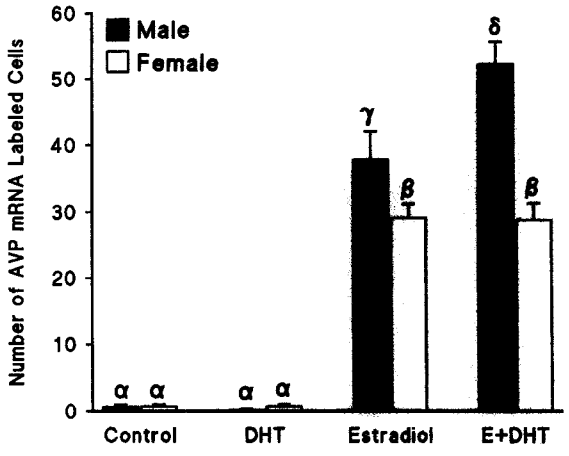


Fig. 6. Differences in the number of cells in the bed nucleus of the stria terminalis that were labeled for VP mRNA in male and female rats that were gonadectomized (control), or gonadectomized and treated with dihydrotestosterone (DHT), estradiol, or a combination of estradiol and DHT (E + DHT). Greek letters indicate significant differences (ANOVA). Bars, means ± SEM. Adapted from De Vries et al. (1994).

VP expression. Estrogen might increase the responsiveness of individual BST and MA cells to DHT treatment by preventing the metabolic inactivation of DHT in the brain, for example (Södersten, 1980). This could explain why DHT implants into the brain, which generate locally high concentrations of DHT, stimulate VP mRNA expression whereas peripheral administration of DHT does not (De Vries et al., 1986, 1994; Brot et al., 1993). Alternatively, estrogen might increase the effectiveness of androgen receptors of VP-producing cells by altering the duration of androgen receptor occupation, as it does in the preoptic area of male rats (Roselli and Fasasi, 1992).

Recently, we have found that virtually all VP-immunoreactive cells in the BST and MA express progesterone receptor immunoreactivity (Auger et al., 1997), suggesting yet another way in which steroid hormones may influence VP expression. The gene that codes for VP has a consensus sequence for a glucocorticoid receptor response element (Mohr and Richter, 1990), which is likely to bind progesterone receptors (Funder, 1997). As gonadectomy reduces progesterone receptor immunoreactivity in VP-ir cells in the BST and MA

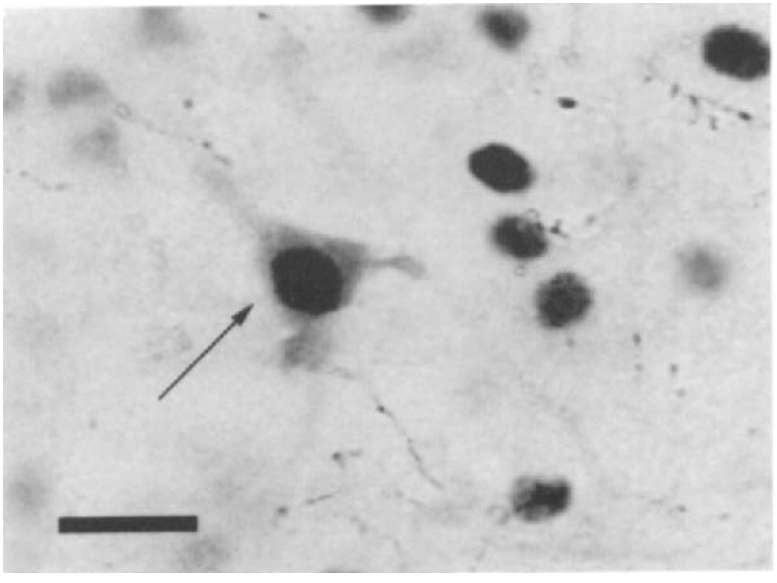


Fig. 7. Colocalization of VP-immunoreactivity (lightly stained cytoplasm) with androgen-receptor-immunoreactivity (darkly stained nuclei) in the medial amygdaloid nucleus. Arrow points at a VP-immunoreactive cell with an androgen receptor-immunoreactive nucleus (bar = 10 μm).

(Auger et al., 1997), gonadal hormones may influence VP expression in the BST and MA by influencing the interaction of progesterone receptors with the VP gene.

Sex differences in VP projections of the BST and MA

In addition to the effects of gonadal steroids on VP expression in adulthood, gonadal steroids determine the sexual differentiation of these projections during development. Males have a much denser VP-immunoreactive innervation of the lateral septum and lateral habenular nucleus from the twelfth postnatal day onwards (Fig. 8) (De Vries et al., 1981). The same difference is also present in other VP-immunoreactive projections of the BST and MA (De Vries and Al-Shamma, 1990). Consistent with this difference, males have about two to three times more VP-expressing cells in the BST and MA than females (Van Leeuwen et al., 1985; Miller et al., 1989b; De Vries and Al-Shamma, 1990; Wang et al., 1993; Szot and Dorsa, 1993; De Vries et al., 1994; Al-Shamma and De Vries, 1996).

The sexual differentiation of the VP projections of the BST and MA appears to depend on gonadal hormones in a similar manner as does the sexual differentiation of reproductive behavior. In rats, gonadal hormones determine sexual differentiation of behavior by influencing the development of specific neuronal systems during a restricted period around birth–often referred to as the critical period (Gorski, 1984; Yahr, 1988; Breedlove, 1992). For example, females given testosterone in the first week after birth will show high levels of masculine sexual behavior after being primed with testosterone in adulthood but low levels of female sexual behavior after being primed with estradiol and progesterone, whereas males that were castrated neonatally will show the reverse pattern (Gorski, 1984). In a parallel fashion, male rats that were castrated neonatally showed fewer VP-immunoreactive cells in the BST and a lower density of VP-immunoreactive fibers in the lateral septum than male rats castrated when they were 3 months of age, even though these rats had been treated with similar levels of testosterone before sacrifice. In fact, the VP-immunoreactive cell numbers and fiber density of neonatally castrated rats did not differ from female rats that were ovariectomized neonatally or at 3 months of age (Fig. 9). This

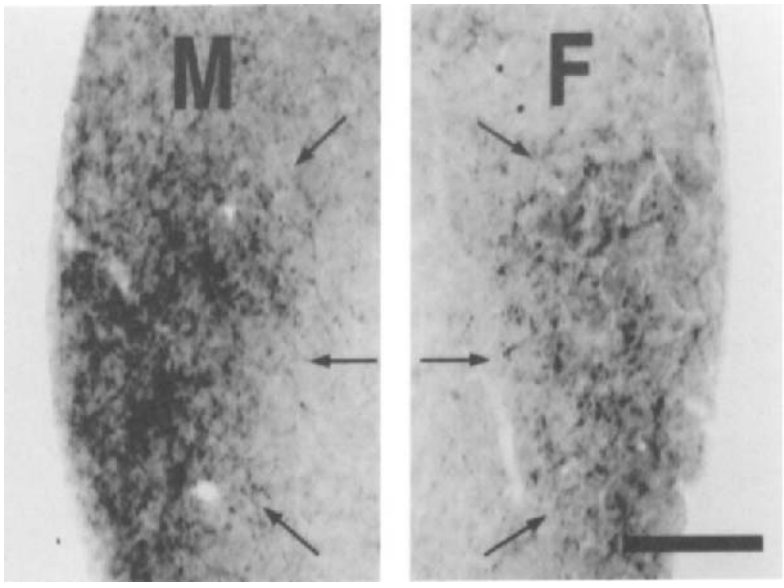


Fig. 8. VP-immunoreactive fibers (arrows) in the lateral septum of a male (M) and female rat (F) (bar = 250 μm).

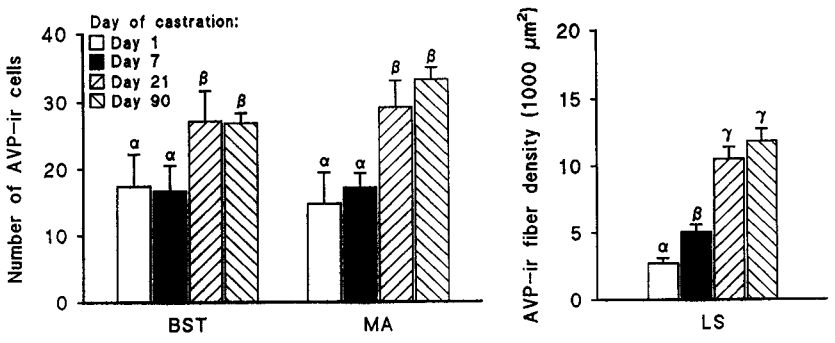


Fig. 9. Effects of castration at 1, 7, 21, or 90 days after birth on the number of VP-immunoreactive cells in the BST and MA and the VP-immunoreactive fiber density in the lateral septum. Greek letters indicate significant differences (ANOVA). Bars, means ± SEM. Adapted from Wang et al. (1993).

suggested that testicular secretions after birth permanently influence the development of the VP-immunoreactive projections of the BST. Indeed, testosterone propionate given at the 7th postnatal day significantly raised VP-immunoreactive fiber density in the lateral septum of neonatally gonadectomized male and female rats, and fully restored the number of VP-immunoreactive cells in the BST of neonatally castrated males but not of females (Wang et al., 1993).

The sex differences in VP expression seen in adult rats even after treatment with similar levels of testosterone (De Vries and Al-Shamma, 1990; Wang et al., 1993) could have been caused by differences in testosterone metabolism. The BST of male rats has higher levels of aromatase, the enzyme that catalyzes the aromatization of testosterone into estradiol, than the BST of female rats (Roselli, 1991). Therefore, testosterone may stimulate BST cells more effectively in male than in female rats. However, a sex difference in testosterone metabolism cannot be the only factor underlying sex difference in the VP projections of the BST and MA either, because sex differences in VP mRNA expression remained present if rats were treated with similar levels of the testosterone metabolites estradiol and DHT instead of testosterone (De Vries et al., 1994). These treatments with testosterone metabolites suggested that sex differences in androgen responsiveness contribute to sex differences in VP expression, because DHT boosted VP mRNA expression when given together with estradiol in males but not in females (Fig. 6). A higher number of androgen receptors in the BST in males than in females (Roselli, 1991) may explain why DHT was effective in males but not in females.

Development of VP cells in the BST and MA

To understand which particular cellular features make the developing VP-immunoreactive cells responsive to the differentiating influences of gonadal hormones, we are studying the early development of these cells. This is complicated by the late onset of VP mRNA expression in the BST and MA, which in males becomes detectable at postnatal days 3 and 5, and in females at postnatal days 21 and 35, respectively (Szot and Dorsa, 1993), making it difficult to identify future VP-immunoreactive cells during the period that gonadal steroids influence their sexual differentiation. Using the thymidine analog bromo-2-deoxy-5-uridine (BrdU) as a birth marker, we found that most VP-immunoreactive cells are born on embryonic days 12 and 13 (the day that sperm plugs are found being embryonic day 1). This places the VP cells of the BST and MA in the earliest cohort of cells that will form the adult forebrain (Al-Shamma and De Vries, 1996). This early birth date sets VP apart from surrounding cells, the majority of which is born on embryonic days 14–16 (Fig. 10).

This difference in cell birth between VP cells and other cells in the same area may play a role in BST

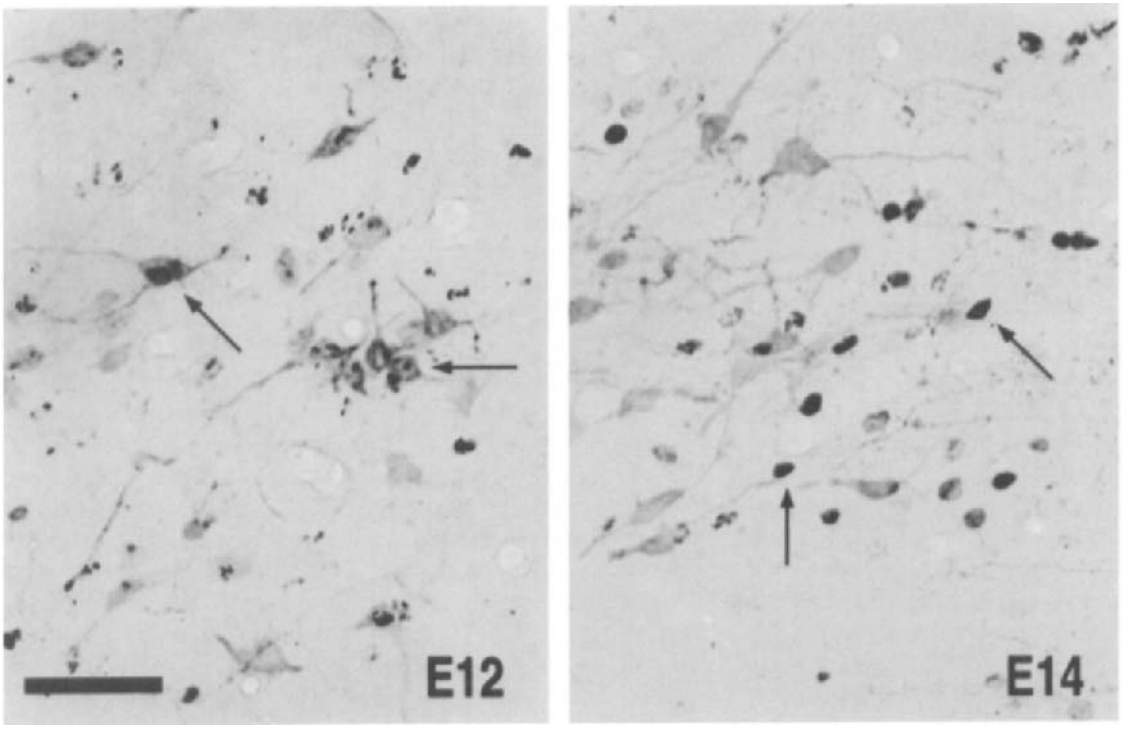


Fig. 10. Cells in the bed nucleus of the stria terminalis labeled by injecting the cell birth marker bromodeoxyuridine into pregnant rats on embryonic day 12 (E12) and embryonic day 14 (E14) in sections that were immunostained for bromodeoxyuridine (black nuclei) and VP (lightly stained cytoplasm). Note that injections on E12 label nuclei of cells that are immunoreactive for VP (arrows), whereas injections on E14 label mostly the nuclei of other cells (arrows) (bar = 25 μm).

cells developing a VP-expressing phenotype. This early birth date may also make VP cells more susceptible for sexual differentiation than they would have been if they would have emerged later. There is a larger sex difference in the number of VP-immunoreactive cells born on day 12 than in the number of cells born on day 13 (Al-Shamma and De Vries, 1996) suggesting that cells born earlier are more likely to sexually differentiate than cells born later. It is unlikely that a higher cell birth rate in the BST of males causes the sex difference in the number of VP-expressing cells because hormonal manipulations of neonates can still influence the sexual differentiation of the VP-immunoreactive cells in the BST and MA (Wang et al., 1993). It is more likely that in males and females equal numbers of cells are born that may express VP in adulthood. During sexual differentiation, these numbers may change, either by programmed cell death, which plays a role in the sexual differentiation of the spinal cord (Breedlove, 1992), or by the cells taking on different phenotypes.

There is, in fact, a distinct possibility that differentiation of phenotype underlies the sexual differentiation of VP cells in the BST and MA. VP in the BST and MA is co-expressed with the neuropeptide galanin (Miller et al., 1993; Planas, 1995a). Virtually all VP neurons in the BST and MA of male and female rats express galanin mRNA but only a subset of the galanin neurons in the BST and MA express VP. Although galanin in the BST and MA is also regulated by gonadal hormones (Miller et al., 1993; Planas et al., 1994a), there is no sex difference in the total number of galanin-expressing cells in the BST and MA (Planas et al., 1994b). However, there are sex and regional differences in the coexpression of VP and galanin

in the BST that can account for the sex difference in VP cell number. Whereas males and females have a similar level of coexpression of galanin and VP in the lateral subdivision, males have about two times as high a percentage of galanin cells in the medial subdivision that also express VP (Planas et al., 1995b). These findings suggest that a subset of galanin neurons in the BST and MA are programmed to co-express VP independently of the perinatal hormonal milieu, but also that, in males, an additional set of galanin neurons is organized during the critical period of sexual differentiation to coexpress VP. Recently, we have demonstrated that most galanin-ir neurons in the BST and MA are born, like VP-immunoreactive neurons in these areas, on embryonic days 12 and 13. This supports the idea that all galanin-ir neurons in the BST and MA belong to a single set of neurons (Han and De Vries, 1998). Therefore, in the perinatal period, gonadal hormones may influence the capacity of galanin neurons to synthesize VP rather than influencing the survival and/or proliferation of VP neurons per se.

Function of BST and MA projections

The sexual dimorphism and steroid responsiveness of the VP projections of the BST and MA do not mean that these projections only control sexually dimorphic and steroid-responsive functions. The VP projections of the BST and MA innervate subcortical forebrain areas such as the lateral septum, lateral habenular nucleus, the ventral pallidum, midbrain areas such as the midbrain central grey, the ventral tegmental area, dorsal raphe, and hindbrain areas such as the locus coeruleus (De Vries and Buijs, 1983; De Vries et al., 1985). These areas have been implicated in a staggering number of functions, only some of which show very obvious sex differences (Paxinos, 1995). The multimodal nature of these target areas suggests that VP may influence many functions simultaneously and that, consequently, changes in gonadal hormone levels would affect the regulation of these functions simultaneously as well. This suggests that perhaps one of the most important functions of VP pathways of the BST and MA is to orchestrate changes in brain physiology when gonadal hormone levels fluctuate. Here we will discuss the evidence for involvement of VP in specific functions that may shed light on the functional significance of the steroid influences on the VP projections of the BST and MA.

One function influenced by these projections may be male sexual behavior. Lesion studies have implicated the BST and MA in male sexual behavior (Harris and Sachs, 1975; Valcourt and Sachs, 1979). A potential involvement of the VP-immunoreactive projections of the BST and MA in male sexual behavior fits with the similarities in the effects of testosterone metabolites on male sexual behavior and on the density of the VP-immunoreactive innervation of the lateral septum (Baum and Vreeburg, 1973; Larsson et al., 1973). Treatment of rats with a centrally acting VP analogue, in fact, reversed the decline of copulatory patterns following castration and stimulated the motivational aspects of male sexual behavior (Bohus, 1977). However, injections of VP directly into the lateral septum did not influence male sexual behavior (Koolhaas et al., 1991).

Lesion and stimulation studies have implicated areas innervated by the sexually dimorphic VP fibers in female sexual behavior as well (Nance et al., 1974; Zasorin et al., 1975). In fact, VP stimulates female sexual behavior and a $V1_a$ receptor antagonist inhibits it when injected into the cerebral ventricles (Södersten et al., 1985). However, a direct link between the VP innervation of the lateral septum and female sexual behavior has never been tested.

A more convincing argument can be made that the VP projections of the BST and MA influence aggressive behavior. Intermale aggressive behavior, for example, declines gradually after castration, as does male sexual behavior (DeBold and Miczek, 1984), but injections of VP into the lateral septum or the MA can reverse this decline (Koolhaas et al., 1990, 1991). The higher level of VP in males and the decrease in VP production after castration appears to correlate with known dimorphisms, and steroid responsiveness of aggressive behavior and male and female sexual behavior. However, septal VP has been implicated in a number of other functions for which this relationship is less obvious.

Fever reduction, which is the first function clearly linked to septal VP (Cooper et al., 1979), does not show dramatic sex differences. Fever-reducing effects of VP and fever-enhancing effects of V_a receptor antagonists injected specifically into the ventral septal area have been demonstrated repeatedly for a variety of mammals including rats (Kasting, 1989). The ventral septal area probably receives its VP-immunoreactive innervation from the BST. Like other projections of the BST and MA, this innervation is denser in males than in females (De Vries and Al-Shamma, 1990) and disappears after castration (De Vries et al., 1985). It is also strongly reduced after lesioning the BST (De Vries and Buijs, 1983). In addition, electrical stimulation of the BST changes the electrical activity of ventral septal neurons in a similar manner as does exogenous application of VP (Disturnal et al., 1985a,b). Electrical simulation of the BST also attenuates pyrogen-induced fever, presumably by stimulating VP release in the ventral septal area because this effect can be blocked by injecting a V_{1a} receptor antagonist into the ventral septal area (Naylor, 1988). Finally, castration, which presumably reduces VP release in the lateral septum, lengthens pyrogen-induced fever (Pittman et al., 1988).

A recent study has shown sex differences in fever reduction that may be related to the sex differences in the VP projections of the BST and MA (Chen et al., 1997). Centrally administered prostaglandin E_2 induced higher fevers in females than in males. This difference may be related to a differential stimulation of VP release in the ventral septal area, because prostaglandin E_2 infusion increased VP release in males but not in females. In addition, infusion of a V_{1a} receptor antagonists increased these fevers in males but not in females, presumably by blocking the effects of the increase in VP release in males.

Another study in which the steroid-responsiveness of the VP projections of the BST and MA has been exploited, focused on the effects of castration on the ability of interleukin-1 to induce not only fever but also 'sickness behavior', which is characterized by increased sleepiness, lethargy, reduced social activities, and reduced food intake. Taking social investigation of juvenile conspecifics as an index of sickness behavior, Dantzer et al. (1991) and Bluthe and Dantzer (1992) showed that castrated rats were more sensitive to the fever-inducing and sickness behavior-inducing effects of interleukin-1 than intact rats.

This increased sensitivity in castrated rats may be related to the absence of VP in the BST and MA projections, because VP attenuates the effects of interleukin 1 more effectively in castrated than in intact rats. Presumably endogenous release of VP in the intact rats might already have attenuated the effects of interleukin-1. Indeed, treatment with a V_{1A} receptor antagonist potentiated the effects of interleukin 1 in intact but not in castrated rats, suggesting that endogenous release of VP in intact rats attenuates the fever-inducing and sickness behavior-inducing effects of interleukin-1.

Injections of VP and its V_{1A} antagonist into the lateral septum suggest that septal VP also enhances another social behavior, i.e., social recognition memory (defined as the ability of rats to recognize conspecifics that they had previously investigated; Dantzer et al., 1988). However, septal VP may enhance social recognition memory in males only. Injections of a V_{1A} receptor antagonist block social recognition memory in males but not in females. Because social recognition memory is not impaired in females, it appears to have different neurochemical underpinnings than it has in males. The same is true for long-term castrated males, which also do show no decrease in social recognition memory after injections of a V_{1A} receptor antagonist. Because social recognition memory is not impaired in long-term castrated males either, the decrease in septal VP appears to have been compensated (Bluthe et al., 1990; Bluthe and Dantzer, 1992).

The effects of VP on intermale aggression and on social recognition memory represent opposite ways in which the sexually dimorphic VP projections may contribute to sex differences in centrally regulated functions. In case of intermale aggression, they appear to stimulate sex differences in behavior as this behavior is more common in males than in females. In case of social recognition memory, these projections may actually do exactly the opposite, that is, they may prevent sex differences in this behavior, possibly by compensating for physiological and hormonal differences between males and

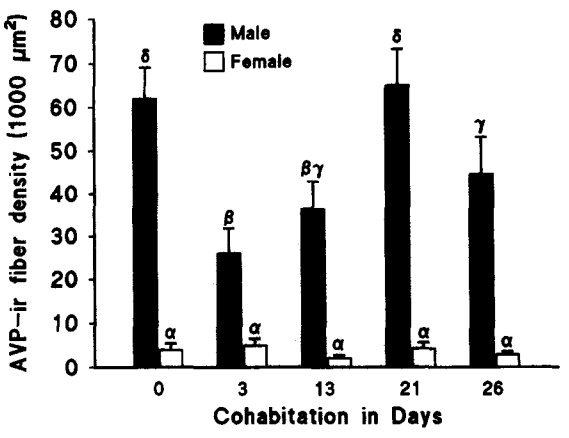


Fig. 11. VP-immunoreactive fibers in the lateral septum before (0) and after 3, 13, 21 days of cohabitation in males and females and at 6 days postpartum (28). Greek letters indicate significant differences (ANOVA). Bars, means ± SEM. Adapted from Bamshad et al. (1994).

females. Although the need for this type of compensation is not instantly clear for social recognition memory, we have recently studied a case for which this need is more clear, namely biparental behavior in prairie voles.

In most mammalian species, changes in ovarian hormone levels that are induced by pregnancy, prime the brain of females for maternal behavior (Bridges, 1990). Therefore, in species in which males as well as females participate in parental care, paternal behavior must be induced through different mechanisms than is maternal behavior. In addition, neural circuits involved in parental behavior may be affected in a sex-specific manner, because parental males and females face different physiological challenges; for example, males do not lactate. Such circuits may need to differ to guarantee a similar behavioral output between males and females.

In prairie voles (*Microtus ochrogaster*), sexually naive males show spontaneous parental responsiveness. Mating, however, considerably increases paternal responsiveness (Bamshad et al., 1994). Mating also reduces the density of the VP-immunoreactive projections of the BST and MA to, for example, the lateral septum in prairie vole males (Fig. 11) (Bamshad et al., 1994) while it increases VP messenger RNA levels in the BST (Wang et al., 1994b). The drop in VP-immunoreactive fiber density in the lateral septum after mating may, therefore, reflect an increase in VP release. This presumed increase in VP release may underlie the increase in paternal responsiveness, because injections of VP into the lateral septum of naive male prairie voles stimulated paternal responsiveness while injections of a V_{1a} receptor antagonist blocked it (Wang et al., 1994a). Mating did not induce any changes in the VP system of females, which suggests that the sex difference in the VP innervation of the lateral septum may indeed function to allow males to show a similar behavioral response to pups as do females. More details on these comparative studies can be found in the chapter by Wang et al. in this book and in De Vries and Villalba (1997).

Conclusions

With the increasing number of functions attributed to VP in the brain (De Wied et al., 1993), the question as to how VP regulates several functions simultaneously becomes increasingly more pressing. Answers to that question must make it easier to understand how the sexually dimorphic VP projections of the BST and MA can promote sex differences in some functions (for example, aggressive behavior) and prevent them in others (for example, social recognition memory and parental behavior). In order to elucidate how neuropeptide systems may play both roles at the same time several conditions must be met: one should have identified which neural systems generate sexually dimorphic functions and which neural systems generate non-sexually dimorphic functions; one should also have determined where these systems overlap and how the function of each of these systems influence the functions of the other systems. Ideally one would to integrate answers to these questions with an understanding of the functional consequences of the colocalization of VP with other peptides such as galanin. Meeting these conditions appears a Utopian goal. However, increasing our understanding of the neural basis of integration of behavior and homeostatic functions has some medical urgency, as it will help us

understand how behavioral disorders are often linked to abnormalities in physiology.

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CHAPTER 1.2

Functions of the perikaryon and dendrites in magnocellular vasopressin-secreting neurons: New insights from ultrastructural studies

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Magnocellular hypothalamic neurosecretory neurons secreting vasopressin or oxytocin provide a robust model system for the investigation and understanding of many aspects of peptidergic neuronal function. Many of their functions and the cellular organelles involved are well understood. However, recent ultrastructural studies have thrown new light on various aspects of magnocellular neurosecretory function which have not previously received much attention. This review concerns two of these: the effects of mutations in the vasopressin gene on the handling of the translated peptide by the rough endoplasmic reticulum; and the role of the magnocellular dendrites in the production, secretion and localisation of peptides. Investigation of the synthesis of proteins derived from vasopressin genes which have undergone various mutations has at the moment provided more answers than questions: Why do some abnormal products accumulate as masses of peptide in the rough endoplasmic reticulum while others do not? Why do accumulations in humans appear to be damaging to the neurons while those in the rat do not? Investigations of the role of dendrites in the production and release of peptides show that the dendrites have all the machinery needed for protein translation and appear to synthesize locally proteins required for dendritic function. Of particular interest is the possibility that various transmitter receptor proteins could be synthesized in the dendrites close to the synapses in which they become localized. Precisely how such membrane proteins are inserted into the synaptic complex is, however, unclear, because the most part of the dendrites lack any form of the Golgi packaging organelle that can be recognised as such either by immunocytochemistry or electron microscopy. Better established is the ability of magnocellular dendrites to secrete either vasopressin or oxytocin in response to a variety of stimuli including sex steroids. This local release of peptide into the magnocellular nuclei has important but as yet incompletely defined effects on the functioning of the neurons.

Introduction

The general light microscopic and ultrastructural features of magnocellular neurons are well known and have been extensively reviewed (e.g. Morris et al., 1987; Theodosis and Poulain, 1993; Hatton, 1997). Recent advances in our understanding of the functions of vasopressin (VP) neurons in which ultrastructural studies have played a significant part include new insights into: (a) the way in which the rough endoplasmic reticulum (rER) handles the gene products, in particular products of mutated VP genes; and (b) the role of the magnocellular dendrites in the production, localisation, and release of peptide products. In both these areas, the insights have benefitted from a combination of immunocytochemistry and in situ hybridization combined with the precise structural localization provided by electron microscopy. This review concentrates on recent insights provided by structural studies, and on the cell bodies and dendrites in the hypothalamus rather than the axonal terminals in the neural lobe.

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New insights into endoplasmic reticulum functions from mutations of the VP gene

Vasopressin (and oxytocin) neurons in the homozygous Brattleboro rat (BB) (Valtin, 1982; Valtin et al., 1992) in which a single base deletion in the neurophysin coding region of the VP gene prevents secretion of VP, are subject to a continuous if fluctuating hyperosmotic stimulus as a result of their diabetes insipidus. The VP gene is located back to back with the OT gene on a single chromosome separated by an intervening sequence which differs in length among species (Richter, 1986; Mohr et al., 1988; Schmitz et al., 1991); both alleles of the VP gene are expressed (Valtin et al., 1974).

It has been known for many years that BB magnocellular neuron cell bodies are hypertrophied (see Morris, 1982; and below for dendrites) and that the endoplasmic reticulum and Golgi apparatus differs markedly in its arrangement from that in the normal parent (Long–Evans, LE) strain. Ultrastructural studies show the endoplasmic reticulum in BB rats takes the form of many small saccules rather than the parallel lamellae present in most VP cells in LE rats; the Golgi apparatus is also dispersed in multiple small stacks throughout the cytoplasm rather than in the perinuclear region. As in LE rats, very little proteinaceous material can be detected in the rER cisternae of most VP neurons, although light microscopic studies have suggested some translation of VP occurs in BB rats (Guldenaar et al., 1986). The mutant VP mRNA is known to be poorly translated (Schmale et al., 1984) and it has been suggested that it is trapped in rER-bound ribosomes by the polylysine tail resulting from the lack of a stop codon and subsequent translation of the poly-A tail, but the extent to which this might be associated with the altered arrangement of the rER and Golgi is unknown. It is useful, therefore, to look at another naturally occurring mutant in which the VP neurons are subject to a similar osmotic stress, but in which the VP gene is normal. Mice with hereditary nephrogenic diabetes insipidus (di/di) provide such a model (Valtin et al., 1992). In such animals, the VP cells are hypertrophied, but the rER and Golgi apparatus have a much more normal arrangement and the cells produce large numbers of VP-containing neurosecretory vesicles (Morris et al., 1993). It will therefore be important in the future to localize precisely the mutant VP mRNA in the BB neurons.

Not all BB magnocellular neurons fail to produce detectable amounts of VP or its associated neurophysin (NP) or glycopeptide (GP). Occasional isolated VP neurons contain immunocytochemically detectable amounts of all three products and the presence of the glycopeptide in particular demonstrates clearly that the frame shift in the BB VP gene has been corrected. Such cells were first detected by Richards et al. (1985) but it has been the work of van Leeuwen and his colleagues (Van Leeuwen et al., 1989; Van Leeuwen and Van der Beek, 1991) that has advanced our understanding of this phenomenon. They have shown that the incidence of VP-producing cells increases with age until 23 months (Van Leeuwen et al., 1994) and that the reversion is the result of many different, presumed somatic mutations (presumed only because all the studies involve analyses of mRNA rather than genomic DNA), which either remove or add bases to correct the reading frame (Evans et al., 1994). There is light microscopic evidence that, in these neurons, the VP and GP are transported to the neural lobe and are therefore presumably packaged normally (Van Leeuwen et al., 1994) although our own electron microscopic studies did not detect secretory vesicles in such revertant cells (Pow et al., 1992). The somatic mutations affect primarily two hot-spots in the VP gene (Evans et al., 1994).

A second group of neurons in BB rats also produce detectable amounts of VP (Pow et al., 1992) but for a very different reason. In these animals, a somatic cross-over mutation has occurred between the VP gene and the adjacent, highly homologous oxytocin (OT) gene, both of which are strongly stimulated. Analyses of hypothalamic mRNA in BB rats shows that the cross-overs occur almost exclusively in the 95% homologous B exons (Mohr et al., 1994). The peptide that results from 5′VP-OT3′ hybrid mRNA accumulates to form large peptide aggregates which fill many of the rER cisternae in such cells and are immunoreactive for VP and for the

OT-associated NP (OTNP). None of the neurosecretory vesicles in which VP is packed in LE rats or Brattleboro heterozygotes can be detected, and it therefore appears that the hybrid peptide cannot be packaged and does not leave the rER by the regulated secretory pathway. Cells with accumulations of hybrid VP-OTNP peptide increase with age like those with frame-shift reversions, but are less frequent (up to 24 per hypothalamus compared to 130 per hypothalamus). In the autosomal dominant form of hypothalamic diabetes in humans, the most common mutation is again in the neurophysin-coding region (Robertson, 1995; Repaske and Browning, 1994; Bahnsen et al., 1992; Nagasaki et al., 1995; Rauch et al., 1996; Ueta et al., 1996; Gagliardi et al., 1997). Aggregates of peptide do form in the rER in this condition, and are thought to be toxic to the cells (Ito et al., 1997). In this they differ from the peptide aggregates in the rat, in which there is no evidence that the aggregates damage the cell or affect their viability.

Although there is increasing understanding of the role of chaperone systems in endocrine cells and endocrine disorders (Kim and Arvan, 1998), why the peptide should accumulate is something of a mystery, for a number of reasons. First, analyses of hypothalamic mRNA from BB rats shows that both 5′VP-OT3′ and 5′OT-VP3′ hybrid mRNAs are present, in roughly similar amounts (Mohr et al., 1994) but cells with accumulations of peptide immunoreactive for OT and VP-associated NP and GP have never been detected. Second, similar hybrid mRNAs can be detected in both normal LE rats and in untreated di/di mice, but so far no cells with accumulations of peptide in the rER cisterns have been detected in these animals (Budd and Morris, 1997; Epton and Morris, unpublished observations).

Morphometric analysis of the peptide aggregates in BB rats shows that they are of a similar size in both young (3–6 month) and old (2–3 year) animals (Budd and Morris, 1997). This strongly suggests that some mechanism is able to remove the hybrid peptide from the rER and to degrade it. We are currently examining whether the ubiquitin system, which is known to be able to extract misfolded proteins from the rER and degrade them (Hiller et al., 1996; Sommer and Wolf, 1997), plays a role in this. Whatever the mechanism, it would appear that the amount of peptide aggregate visualised with the electron microscope represents a balance between the rate of production and the rate of removal. This could explain why aggregates are absent in LE rats in which the stimulation of the VP gene is much lower than in BB rats, but it does not explain why the OT-VPNP peptides do not accumulate in BB rats. Certainly it is not that aggregates of peptide cannot accumulate in OT-secreting neurons because aggregates of peptide with the normal phenotype of OT precursors (OT and OTNP) can readily be detected in the cells and are increased in amount by oestradiol stimulation (Pow et al., 1991; and see below). Also, it is quite unclear why peptide aggregate-containing cells are 6-fold more common in the supraoptic nucleus than in the paraventricular nucleus, when there is no obvious difference between the structure and function of the magnocellular neurons in the two nuclei.

Because the di/di mouse has diabetes insipidus as severe as that of the BB rat, we expected to find that the incidence of cells containing aggregates of hybrid VP-OT peptide would be similar to that found in the BB rat. However, an exhaustive search of serial sections through the hypothalamus of aged di/di mice failed to reveal a single cell with the interference-contrast microscopic appearance of cells with rER peptide aggregates. Noting that, in di/di mice, the oxytocin neurons do not appear to be stimulated (Morris et al., 1993) whereas OT cells in the Brattleboro rat are hypertrophied to the same extent as the VP neurons, oestradiol was administered to a group of di/di mice to stimulate the production of OT. This resulted in the appearance of a small number of neurons with aggregates of rER peptide, but again all aggregates studied to date have contained the VP-OTNP type of hybrid peptide. Analyses of the mRNA in di/di mice, like those in BB rats, has shown the presence of both 5′VP-OT3′ and 5′OT-VP3′ hybrid mRNAs (Epton and Morris, unpublished observations). This, coupled with the finding of hybrid mRNAs in LE rats, shows clearly that the somatic hybridizations are not a function of any difference specific to the Brattleboro rat, neither are the somatic mutations restricted to one species since these somatic hybridizations occur in both rat and mouse, and the

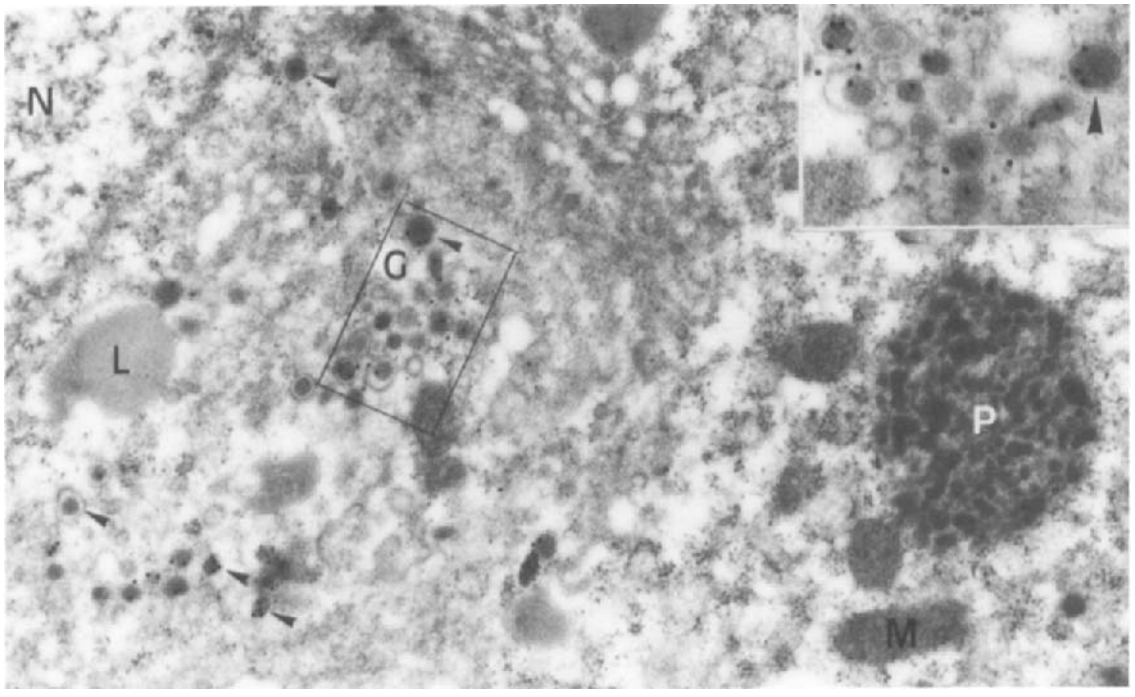

Fig. 1. Part of a vasopressin magnocellular neuron from the supraoptic nucleus of a nephrogenic diabetes insipidus (*di/di*) mouse implanted with an oestradiol-releasing pellet for 10 days. An aggregate of hybrid peptide (P) is present in an endoplasmic reticulum cistern. Typical 160-nm diameter neurosecretory vesicles (arrowheads) are also present in the perinuclear Golgi zone (G). The insert (outlined on main micrograph) shows a higher magnification of the immunogold-identified vasopressin-associated neurophysin in the secretory vesicles. N, nucleus; L, lysosome; M, mitochondrion.

frame-shift reversion somatic mutations occur in rat and human (Evans et al., 1996).

It is as yet unclear why there should be many fewer cells with rER accumulations of hybrid peptide in di/di mice, but one possibility is that the LINE sequence that links the VP and OT genes in the mouse (3.5 kB) is much shorter than that in the rat (11 kB) and that this reduces the chances of cross-over occurring. One aspect of rER function that the aggregate-containing cells in di/di mice does show clearly, however, is that the presence of peptide aggregates in the rER does not prevent the normal packaging into secretory vesicles of the normal gene product. Fig. 1 shows a neuron in a di/di mouse in which a large aggregate of hybrid VP-OTNP peptide is present in the rER and numerous secretory vesicles with cores immunoreactive for VP and VPNP are present in the Golgi region.

This strongly suggests a regional specialisation in the function of the rER. This is consonant with ultrastructural evidence that the frame-shift revertant mRNA is translated in particular regions (Guldenaar et al., 1986) and that dynorphin and VP are translated in different regions of the rER (Van Leeuwen, 1992). The rER may also have a function in the translation of proteins which lack a signal sequence but nevertheless have a membrane-associated role. For example, Trembleau and Bloom (1996) have recently shown that mRNA encoding the GαS protein which interacts with many membrane-located G-protein-coupled receptors is associated with the outer aspect of rER lamellae, and is segregated anatomically from mRNA coding for VP. Likewise Shioda et al. (1997) show similar localisation of the α_4 subunit of the neuronal nicotinic acetylcholine receptor. It therefore appears that we have a lot to learn about

specialisation and localisation of translation functions of the endoplasmic reticulum, and in the way misfolded or otherwise abnormal peptides are handled.

Role of dendrites of magnocellular VP neurons in the synthesis and localisation of proteins

It is well known that, unlike axons, well-defined elements of protein synthetic machinery extend into the dendrites of most neurons, but the precise functions of these organelles and any differences in function from the similar organelles located in the perikaryon is little understood. Magnocellular VP neurons provide a useful model system in which to investigate these questions. They are among the largest neurons, they synthesize and secrete large amounts of peptide, and they have relatively simple dendritic trees which, in the supraoptic nucleus, are gathered together in the zone immediately beneath the nucleus and above the ventral glial lamina.

The dendrites of magnocellular VP neurons in normal animals contain a core of neurotubules, and substantial amounts of free ribosomes (single or as polysomes) which could translate cytoplasmic proteins such as those needed for neurotubule assembly. Consonant with this, in situ hybridization at light and/or electron microscopic levels shows that ribosomal RNA, transfer RNA and poly(A)-mRNA (Fig. 2) extend well into the middle part of the dendrites; mRNA for MAP2, the dendrite-specific microtubule-associated protein, for nitric oxide synthase, which extends throughout the dendrites of both magnocellular and parvocellular suprachiasmatic neurons (Wang and Morris, 1996a,b), and for calcium-calmodulin-dependent protein kinase II (CaMII kinase) which has numerous functions in the dendrites, are also present (Ma and Morris, 1996, 1997a,b and unpublished observations) and could appropriately be translated into the dendritic cytoplasm. All these are present in increased amounts in BB rats compared to their normal LE controls.

The dendrites also contain substantial amounts of rough and smooth ER and numerous VP-containing neurosecretory vesicles which are often accumulated in dilatations along the length of the dendrite. This raises the possibility that secretory vesicles could be produced in the dendrites as well as in the perikarya. Immunocytochemical detection of the rER enzyme protein disulphide isomerase (Fig. 3) confirms the rER extends well into the dendrites and shows that it extends further into the dendrites of BB rats than LE rats (Ma and Morris, unpublished observations); however, we have been unable to detect medial or trans Golgi-related antigens further than the most proximal part of the dendrites. Similarly, ultrastructural analysis reveals no classical stacks of Golgi lamellae in the dendrites, which suggests that any peptide translated in the dendrites could not be packaged there by the regulated secretory pathway, but would have to pass back to the cell body for packaging in the Golgi apparatus located there. mRNA encoding VP can be detected in the dendrites, and accumulations of OT precursor can occasionally be detected in dilations of dendritic rER (Pow et al., 1992) but we have found no evidence for dendritic production of VP-containing neurosecretory vesicles.

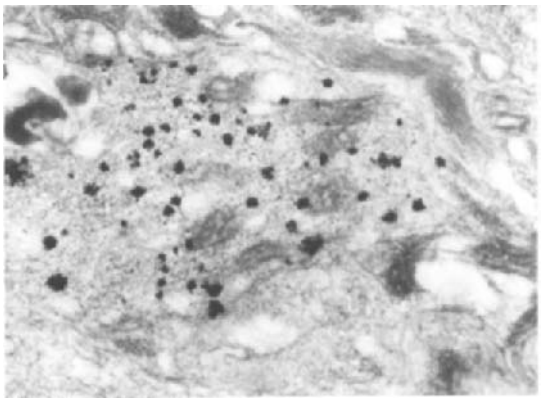

Fig. 2. Electron microscopic in situ hybridization gold-silver detection of Poly(A) mRNA in a magnocellular dendrite in the dendrite-rich region beneath the supraoptic nucleus of a homozygous Brattleboro rat.

A morphometric ultrastructural comparison of the dendrites in homozygous BB rats and in their normal Long–Evans counterparts confirms that the dendrites in BB rats are considerably hypertrophied and also shows that they contain substantially larger amounts of rER and free ribosomes (Fig. 4). However, interestingly, the increase in the amount of rER almost exactly matches the increase in size of the dendrites, so that the proportion of the

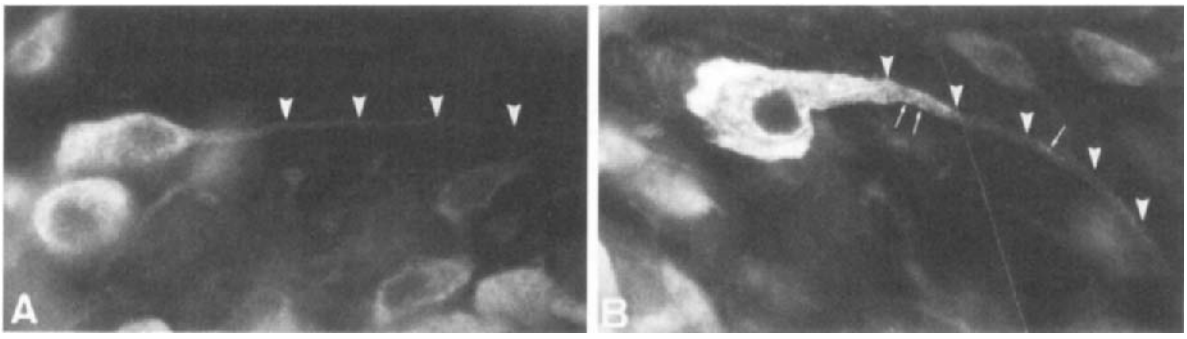

Fig. 3. Immunocytochemical detection of the endoplasmic reticulum-associated enzyme protein disulphide isomerase in magnocellular neurons from the supraoptic nucleus. (A) Neurons from a Long–Evans rat; the immunoreactivity is very strong in the perikarya and extends for some distance into the simple dendrite (arrowheads). (B) Neurons from a homozygous Brattleboro rat; the cytoplasm of the cell is enlarged and intensely immunoreactive; the immunoreactivity extends for a considerable distance into the enlarged dendrite (arrowheads), and in places appears to be concentrated beneath the plasma membrane (fine arrows).

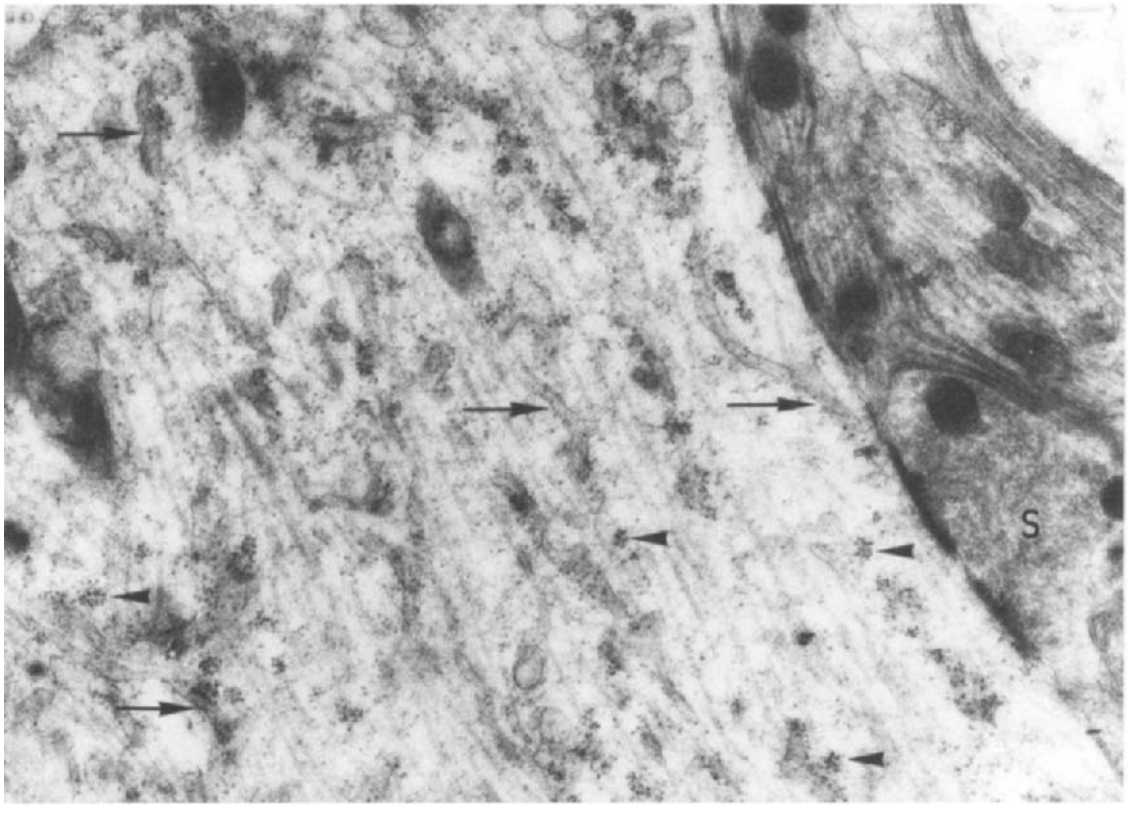

Fig. 4. Part of a dendrite of a magnocellular neuron in the supraoptic nucleus of a homozygous Brattleboro rat. The dendrite is abutted by a synaptic bouton (S) and contains, in addition to many microtubules, many free ribosomes and polysomes (arrowheads) and undilated cisternae of rough and smooth endoplasmic reticulum (arrows), some of which lie very close to the synapse. The dendrite is essentially devoid of neurosecretory vesicles.

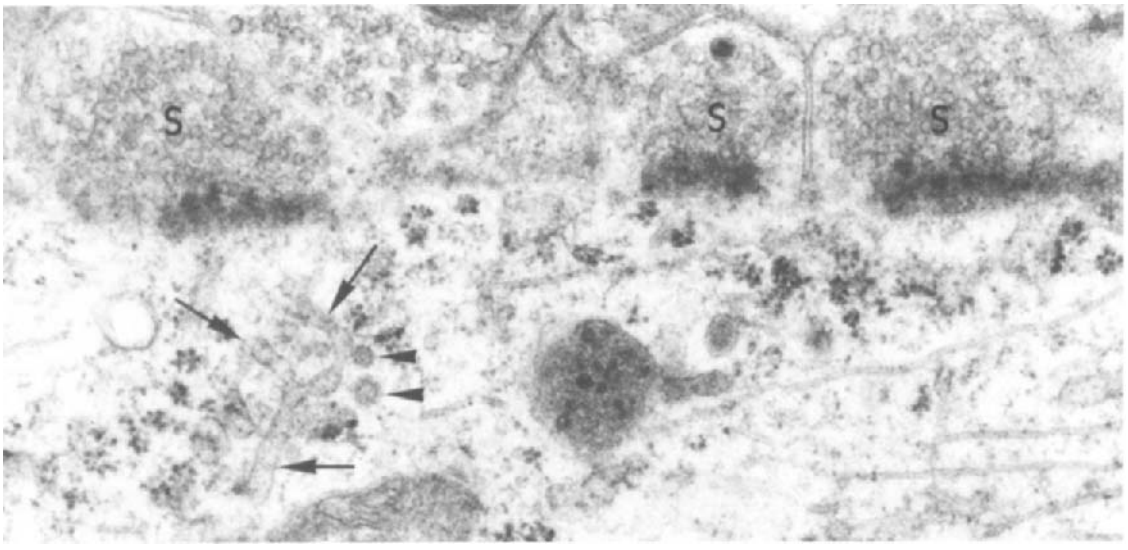

Fig. 5. Peripheral part of a magnocellular dendrite from the supraoptic nucleus of a homozygous Brattleboro rat. Three synaptic boutons (S), with their synaptic contacts cut obliquely, contact the dendrite. In the subsynaptic zone are many free polysomes. At the left is a configuration of smooth endoplasmic reticulum (arrows) and coated vesicles (arrowheads) which could serve as a transport mechanism for peptides produced in dendritic rough endoplasmic reticulum.

dendrites occupied by ER and ribosomes remains similar (Morris et al., 1997). This suggests that the dendritic ER performs functions that are primarily related to the dendrites.

One of the most important dendritic functions is the reception of the majority of the synaptic input. Some polyribosomes are located very close to synaptic sites, and about half the post-synaptic sites have accompanying polyribosomes in the plane of section. Furthermore, in situ hybridization and immunocytochemistry show rRNA, poly(A) mRNA and protein disulphide isomerase to be accumulated in patches beneath the dendritic plasma membrane. This suggests that there may be some very local control on the production and localisation of synapse-related proteins (Steward, 1997). Consonant with this idea, we have localised by light microscopic in situ hybridization, mRNAs for $GABA_A$ and NMDA-R1 in the dendrites. However, these receptors are transmembrane proteins and would be expected to be inserted into the membrane via some sort of Golgi-derived vesicle and, as already noted, there is no light microscopic evidence for Golgi elements in the dendrites, and no classical Golgi stacks present. What we have seen (Fig. 5) is some small tubular-vesicular smooth ER structures, with associated ribosomes, in the cytoplasm immediately beneath certain synapses. It is therefore possible that these provide the morphological substrate for the insertion of proteins into the dendritic membrane.

This could well be important as there is recent evidence for quite rapid alteration of the receptors on magnocellular neurons. This has been shown by elegant analyses of transmitter release (Herbison et al., 1997) and of rapid changes in GABA receptor subunit expression (Fenelon and Herbison, 1996; Brussard et al., 1997) in magnocellular neurons of female rats around the time of parturition.

Role of dendrites of magnocellular VP neurons in the release of peptides and control of the peptide environment of the magnocellular nuclei

It is now clear from both electron microscopic and microdialysis studies that substantial amounts of peptide can be released by magnocellular neurons into the supraoptic and paraventricular

nuclei (Pow and Morris, 1989; Morris et al., 1993; Ludwig et al., 1994). The release of oxytocin into the nuclei has an important function to modify neuronal-glial and synaptic interactions during parturition and lactation (Theodosis et al., 1986). It was to be expected that neurotransmitters which stimulate the release of VP also stimulate its release from the dendrites (see Morris et al., 1993) but it was a surprise to discover that 17β-oestradiol, but not 17α-oestradiol, testosterone, or progesterone caused the rapid (within 5 min) exocytosis of both OT- and VP-containing vesicles from the magnocellular dendrites (Wang et al., 1996). The relation of these morphologic observations to the electrophysiological experiments which show that the neurosteroid pregnenolone sulphate enhances NMDA-induced phasic firing (Wakerley and Richardson, 1997) are not yet clear, but considering that local oestradiol concentrations in the brain are unlikely to change acutely; and that we know too little about the production and role of neurosteroids in the system, it seems likely that these rapid steroid actions will involve modulation of other fast transmitter systems. At least some of the functions of the locally released OT are now clear (Theodosis and Poulain, 1993) but those of VP in the nuclei have been controversial and are only now becoming clearer (Leng and Mason, 1982; Abe et al., 1983; Inenaga and Yamashita, 1985; Dayanithi et al., 1996; Ludwig and Leng, 1997). The mechanism appears to involve both calcium influx (Sabatier et al., 1997) and a retrograde action on the afferent excitation (Kombian et al., 1997). However, it should be noted that a number of these studies involve preparations of magnocellular neurons in vitro and, inevitably during the preparation procedure much of the dendritic tree is lost, with consequences which are at present unknown.

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CHAPTER 1.3

Functional neuroanatomy of the parvocellular vasopressinergic system: Transcriptional responses to stress and glucocorticoid feedback

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This chapter summarizes the regulation of vasopressin (VP) transcription within the parvocellular neurosecretory cells of the hypothalamic paraventricular nucleus in vivo, with special reference to stress-response and glucocorticoid feedback. VP is commonly held as the first and the most potent among the co-secretagogues that act synergistically with corticotropin-releasing factor (CRF-41) to induce adrenocorticotropin (ACTH) from the anterior pituitary in response to various internal and external stimuli. Cellular levels of the primary transcripts of VP and CRF genes, revealed by in situ hybridization histochemistry using probes complementary to intronic sequences, are increased after acute challenges with different time courses. In contrast to the rapid stress-induced upregulation of CRF gene expression, VP transcription shows a delayed increase suggesting different regulatory mechanisms governing the two main ACTH releasing neuropeptides in the parvocellular neurosecretory neurons. With respect of transcription factors that may mediate these effects, besides rapid phosphorylation of the cAMP-response element-binding protein (CREB), VP activation in the parvocellular neurons requires additional newly synthesized factors such as those encoded by immediate-early genes, like c-fos. In addition, it has recently been revealed that glucocorticoid negative feedback during stress, selectively targets vasopressin transcription in the parvocellular neurons that is likely mediated by interaction of glucocorticoid receptors and immediate-early gene products. These data speak for the emerging consensus that VP is the principal factor that imparts situation-specific drive and represents the regulated variable governing hypothalamo-pituitary-adrenocortical axis during stress.

Introduction

There are two neurosecretory cell types within the hypothalamic paraventricular nucleus (PVN) that are capable of secreting arginine vasopressin (VP): the magnocellular VP neurons, and the hypophyseotropic parvocellular neurons. The regulation of VP gene expression and secretion, however, as well as the ultimate role(s) of the released peptide, are vastly different in the magnocellular and parvocellular neurosecretory systems (see Table 1).

The vasopressinergic neurons within the posterior magnocellular subdivision of the PVN give rise to axons which extend through the internal zone of the median eminence to terminate in the neural lobe of the pituitary. Here VP-containing, large dense-core vesicles are released into the systemic circulation upon stimulation by osmosensitive inputs from the lamina terminalis or by viscerosensory afferents relayed via the A1 noradrenergic cells in the lower brainstem (Sawchenko and Swanson, 1983; Cunningham and Sawchenko, 1988). While magnocellular neurons constitutively express vasopressin, as has been revealed by the presence of VP peptide, mRNA, and hnRNA in euhydrated control rats, these intracellular indices are rapidly upregulated in response to osmotic challenges (Burbach et al., 1984; Uhl et al., 1985; Sherman et al., 1986; Young et al., 1986; Herman et al., 1991).

In contrast, perikarya of the parvocellular neurosecretory neurons do not contain detectable amounts of VP immunoreactivity, mRNA, or hnRNA under resting conditions, although VP expression in these corticotropin-releasing factor (CRF)-secreting cells

Table 1
Comparison of magnocellular and parvocellular neurosecretory systems of the hypothalamic paraventricular nucleus

	Magnocellular	Parvocellular
Neuropeptide	VP	CRF-41
Co-secreted peptide	CRF-41	VP
Afferents from	Lamina terminalis A1	NTS, VLM Lamina terminalis V. Hippocampus/BNST Hypothalamus
Efferents through	Zona interna	Zona externa
Efferents to	Neural lobe, pituitary	Portal blood/anterior lobe
Type-2 glucocorticoid receptor	No	Yes
Vasopressin hnRNA	++	No
Vasopressin mRNA	+++	No
Vasopressin peptide	++	No
Adrenalectomy	No change	VP/CRF upregulated
Acute osmotic challenge	VP upregulated	CRF upregulated
Chronic osmotic stimulus	VP upregulated	CRF downregulated
Stress	No change	VP/CRF upregulated

is readily switched on by various acute, repeated, or chronic challenges (Bartanusz et al., 1993; Herman, 1995; Makino et al., 1995; Kovács and Sawchenko, 1996a; Ma et al., 1997).The diverse stimuli which can activate parvocellular CRF neurons may be conveyed by a number of different regions that innervate these cells, including ascending catecholaminergic afferents originating in the solitary tract and ventrolateral medulla, descending innervation from the lamina terminalis, as well as modulatory inputs from the limbic system and from various intrahypothalamic nuclei (Cunningham and Sawchenko, 1988; Sawchenko et al., 1996). Another challenge that elicits expression and release of vasopressin in these parvocellular neurons, as well as an upregulation of CRF, is the removal of the adrenal gland, which normally secretes corticosterone (CORT) to provide the main negative feedback signal in the hypothalamo-pituitary-adrenal (HPA) axis (Kiss et al., 1984; Dallman et al., 1987; Sawchenko, 1987; Herman et al., 1992a; Herman, 1995). These parvocellular neurons are also different from those of VP-positive cells residing in the magnocellular subdivision of the PVN in that they contain type-2 glucocorticoid receptors which mediate a direct inhibitory effect of corticosteroids on neuropeptide gene expression (Kovács et al., 1986; Kiss et al., 1988).

The well-acknowledged functional significance of the parvocellular VP that is co-packaged into small dense-core vesicles and co-secreted with CRF into the hypophyseal portal circulation is to potentiate CRF-induced adrenocorticotropin (ACTH) secretion from the anterior pituitary (Gillies et al., 1982). Upon binding to specific $VP_{1\beta}$ receptors on the corticotrope cells, VP activates the inositol phospholipid cycle and protein kinase C, resulting in the mobilization of Ca^{2+} from intracellular stores and the release of ACTH from the 'rapid-turnover' pool. VP does not stimulate proopiomelanocortin (POMC) transcription as does CRF, but it accelerates the processing of POMC hnRNA, demonstrating another form of functional synergism between these two ACTH secretagogues (for review, see Antoni, 1993).

A clear distinction between the parvocellular and magnocellular vasopressin neurosecretory systems is complicated by several factors. First, there are VP-positive terminals in the external zone of the median eminence, as well as significant amounts of VP in the hypophyseal portal blood, under resting conditions when no signs of ongoing VP synthesis are visible in the parvocellular perikarya

(Plotsky, 1985; Whitnall et al., 1987). It has been demonstrated, however, that magnocellular axons 'in passage' through the median eminence are able to release VP from modified varicosities (Herring bodies) which contribute to the hypophyseotropic neuropeptide cocktail (Holmes et al., 1986; Morris and Pow, 1988; Antoni, 1993), although VP gene expression in the magnocellular neurons is not substantially altered by challenges that target the stress-related parvocellular system. Second, it has recently been shown that single exposure to a stressor can result in long-lasting changes in VP stores in the median eminence, which may serve as a 'stress memory' in adapting further responses to repeated or similar challenges (Schmidt et al., 1995; Schmidt et al., 1996). VP may also be involved in chronic activation, sustained overproduction, and hyperresponsiveness of the HPA axis (Harbuz et al., 1992; Aguilera, 1994), which might contribute to stress-related psychological disorders, such as major depression (Hatzinger et al., 1996; Holsboer and Barden, 1996). These studies led to a shift in the opinion on the role of vasopressin in the HPA axis. Instead of it acting as a simple potentiator of CRF, VP is considered to be a special modulatory factor that may be involved in the stimulus-dependent modulation of the neuroendocrine stress response, as well as in the sustained activation and feedback regulation of the HPA axis. Moreover, VP is a likely substrate for intracellular 'stress memories' to form in parvocellular neurosecretory neurons, producing appropriate sensitization or habituation responses to subsequent challenges.

Technical considerations: the use of intron-specific in situ hybridization histochemistry to asses gene expression

Most of the data discussed in this chapter on changes in neuropeptide gene expression were obtained from in situ hybridization analyses that used an ^{35}S-labeled cRNA probe complementary to the first intron of the VP gene (Herman et al., 1991). This probe hybridizes to intervening sequences within the primary transcript (heteronuclear, hnRNA) before they are excised to form the mature mRNA (Fremeau et al., 1989). Due to the relatively short half-life of the nascent primary transcript, and the fact that its detection is primarily dependent upon synthesis rather than on processing, transportation, and stability, such intron-based hybridization techniques can be used to estimate the actual rate of transcription in individual neurosecretory neurons (Fitzsimmons et al., 1992). This is especially beneficial in measuring vasopressin expression since the stability of its mRNA has been shown to be particularly influenced by activity-dependent and tissue-specific changes in poly-(A)-adenylation (Murphy and Carter, 1990).

Although the timing of hnRNA signal generation correlates well with that of the rate of transcription estimated from nuclear run-on assays, it is important to note that, even under the best of circumstances, the intron-based in situ hybridization technique is just an approximation of stimulus-induced activation of gene transcription, since the signal is also dependent on the splicing rate and the stability of the intermediates (Murphy and Carter, 1990; Herman et al., 1991; Herman, 1995). The major advantage of intron-specific in situ hybridization histochemistry is the high cellular resolution that allows for estimations of gene transcription to be made in individually identified neurons (Herman et al., 1992b; Kovács and Sawchenko, 1996a).

Vasopressin gene expression in parvocellular neurons following an acute challenge

Normal, unstressed rats do not display any discernible VP hnRNA signal in identified parvocellular neurosecretory neurons (Fig. 1). The scattered positive label that has been seen in this region of the PVN could be attributed to ectopic magnocellular neurons, as other vasopressinergic cells in the posterior magnocellular PVN, supraoptic, and suprachiasmatic nuclei show a hybridizable VP hnRNA signal under basal conditions (Herman et al., 1991; Herman, 1995).

A single exposure to ether stress as short as 5 min provoked a marked and transient increase in the VP hnRNA signal specifically in CRF-secreting parvocellular neurons, but not in magnocellular neurons of the PVN (Fig. 1). The first significant increases in VP hnRNA levels in the CRF neurons were seen 1 h following ether exposure, peaked at 2 h, and at 4

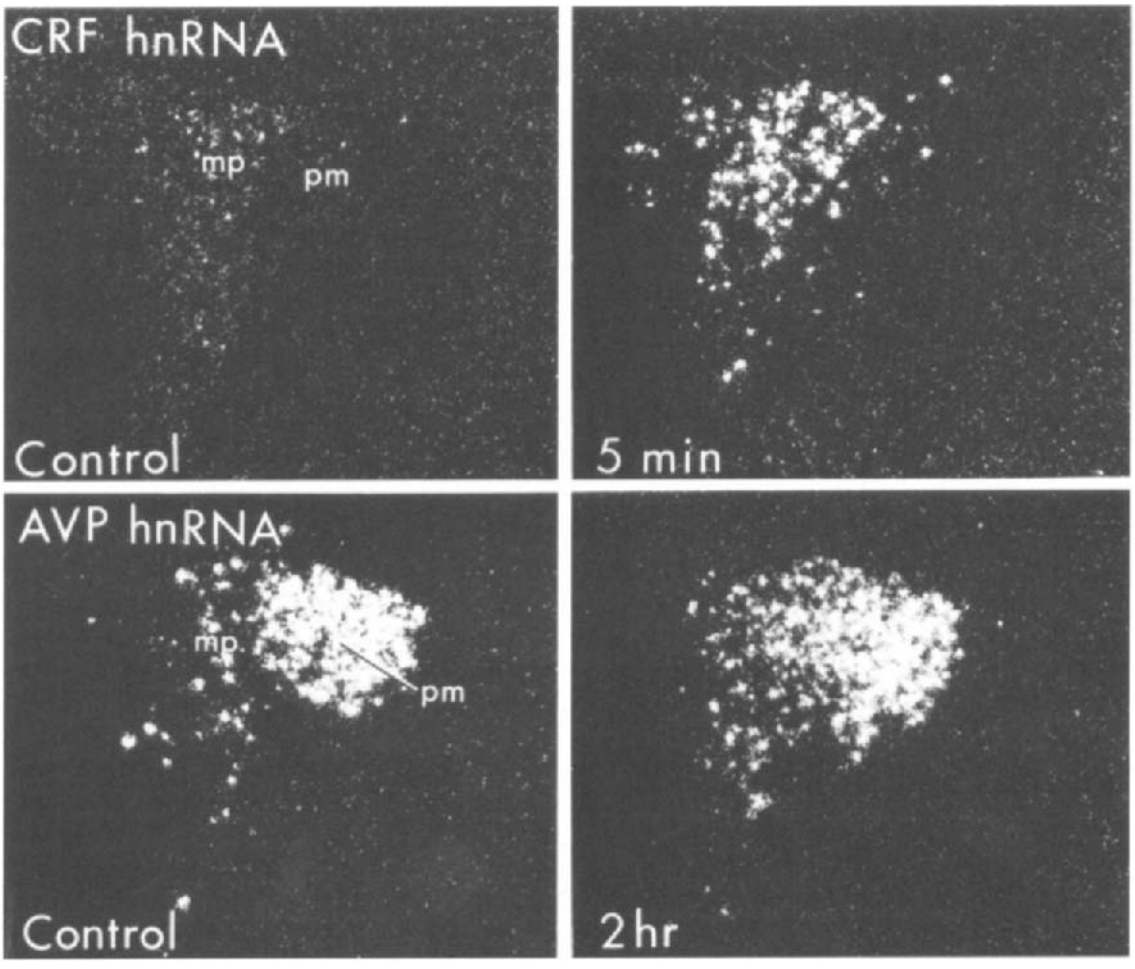

Fig. 1. Dark-field photomicrographs showing ether stress-induced changes in CRF and AVP gene expression in the hypothalamic paraventricular nucleus (PVN). Nuclear hybridization signals were obtained using cRNA probes complementary to the first intron of the vasopressin and the only intron of the corticotropin-releasing factor gene under resting conditions (Control) and at the time of maximal stress-induced response. The maximal responses were: CRF hnRNA at 5 min; AVP hnRNA at 2 h post-stress. Mp, medial parvocellular subdivision; pm, posterior magnocellular subdivision (75×). From Kovács and Sawchenko (1996b).

h post-stress were no longer significantly different from levels seen in unstressed control rats (Fig. 2) (Kovács and Sawchenko, 1996a,b).

Similar time courses in VP gene activation in these cells have been reported after exposure to psychogenic stressors such as an open-field challenge or restraint (Priou et al., 1993; Ma et al., 1997). Compared to the time course of stress-induced CRF hnRNA levels, which peak within 5 min after an acute challenge (Fig. 2), VP gene transcription has a delayed upregulation in the medial parvocellular neurosecretory neurons, suggesting that different mechanisms are involved in regulating the responses of these genes to the same stimulus. Although the functional significance of this difference in the transcriptional response time between these ACTH secretagogues is still unknown, downstream effects on co-packaging and co-secretion of CRF and VP have been suggested, and could have consequences on subse-

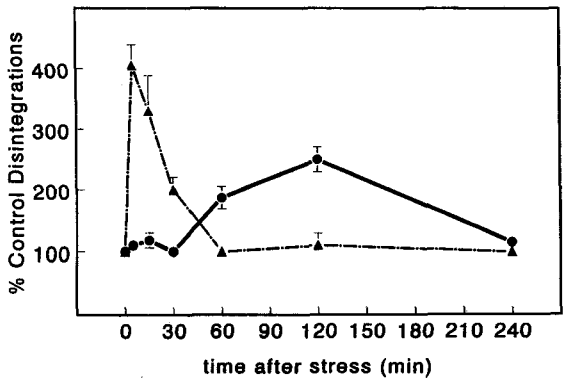

Fig. 2. Relative levels of AVP and CRF hnRNA signals in the medial parvocellular subdivision of the PVN at selected intervals following acute ether stress. From Kovács and Sawchenko (1996a). ▲--▲, CRF hnRNA; ●—●, AVP hnRNA.

quent responses to repeated challenges (Bertini and Kiss, 1991; De Goeij et al., 1991).

Compared to the rapid stimulus-secretion-transcription coupling that has been revealed by data obtained using the intron-specific probe, stress-induced elevations in VP mRNA levels lag considerably behind the stimulus. For instance, significant increases in steady-state VP mRNA levels were not observed until 4 h after restraint, immobilization, hypoglycemia, or hyperosmotic challenges (Lightman and Young, 1988; Bartanusz et al., 1993; Harbuz et al., 1994; Paulmyer-Lacroix et al., 1994). Moreover, relative to the discrepancy between changes in VP hnRNA and mRNA levels in parvocellular neurons in response to an acute challenge, the lag between VP mRNA levels and hnRNA synthesis is even more prolonged in the magnocellular neurons. These cells show an upregulation of intronic VP signal as early as 30–60 min following osmotic stress with no detectable changes in steady-state mRNA levels until 24 h after dehydration or hyperosmotic stimulation (Sherman et al., 1985, 1986; Murphy and Carter, 1990; Herman et al., 1991).

Chronic and repeated challenges can also upregulate VP gene expression along with elevated CRF mRNA levels in parvocellular neurons, a response which has been implicated in the sustained activation of the HPA axis (Kiss and Aguilera, 1993; Aguilera, 1994; Makino et al., 1995). On the other hand, a distinct and pivotal role for elevated VP synthesis and release in chronic inflammatory stress has been suggested by immune-response studies showing an isolated increase in VP synthesis and secretion over that of CRF in parvocellular neurons (Harbuz et al., 1992; Chowdrey et al., 1995).

Factors involved in the induction of the vasopressin gene during an acute challenge

To identify the molecular mechanisms that might be involved in the regulation of neuropeptide gene expression in vivo, the timing of the responses of various transcription factors was compared to changes in VP hnRNA levels in parvocellular neurosecretory neurons. The regulatory region of the vasopressin gene (Fig. 3) contains several consensus sequences that confer responsiveness to a variety of transcription factors (Mohr and Richter, 1990) that have already been shown to be induced/activated in different stress situations (Ceccatelli et al., 1989; Morgan and Curran, 1991; Chan et al., 1993; Ding et al., 1994; Kovács and Sawchenko, 1996a,b; Légrádi et al., 1997).

Included with these transcription factors is the cAMP-response element binding protein, CREB, a constitutively expressed protein which becomes transcriptionally active when it is phosphorylated at Ser^{133}upon various signal transduction mechanisms (Gonzalez and Montminy, 1989; Lalli and Sassone-Corsi, 1994). Immunocytochemical localization studies using affinity purified antisera that specifically recognize the phosphorylated form of CREB revealed the induction of pCREB immunoreactivity within the nuclei of the stress-responsive parvocellular neurons 5 min after ether exposure. Nuclear staining was maximal 15 min post-stress, and thereafter pCREB immunoreactivity progressively diminished until it was no longer detectable 2 h after ether exposure (Kovács and Sawchenko, 1996a; Légrádi et al., 1997). This rapid and transient phosphorylation of CREB is contemporaneous with stress-induced transcriptional activation of the CRF gene, yet precedes the maximal induction of vasopressin hnRNA expression by 1.5–2 h. This lag is surprising since the 5′ region of the vasopressin

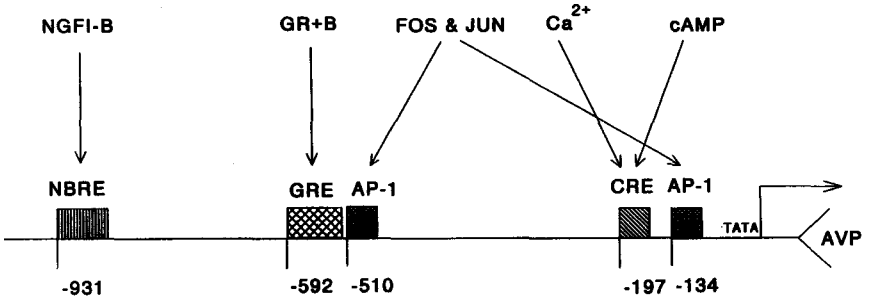

Fig. 3. Demonstrated and potential DNA binding sites (response elements) on the 5′ region of the vasopressin gene. AP-1, activator protein-1 binding site; CRE, cAMP/Ca^{2+} response element; GRE, glucocorticoid response element; NBRE, NGFI-B response element; TATA, TATA box. B, corticosterone.

gene contains several functional CREs (cAMP response elements) (Mohr and Richter, 1990), and activators of adenylate cyclase enhance VP transcription in homologous and heterologous expression systems in vitro (Verbeeck et al., 1990; Pardy et al., 1992), as well as in primary cultures of fetal rat hypothalmi (Hu et al., 1992).

The delay between transcriptional activation of the CRF and VP genes indicates that they are not controlled by a common stimulus-transcription coupling mechanism and factors other than pCREB may be required for transcriptional initiation of the VP gene. Some potential candidates include proteins encoded by immediate-early genes (IEGs), such as c-fos and NGFI-B which are induced in parvocellular neurosecretory cells in response to various challenges (Chan et al., 1993). In general, elevation of IEG mRNAs occurs within 30 min after an acute challenge, with maximal protein expression lasting between 90 and 120 min. The involvement of IEG-encoded transcription factors in the stress-induced regulation of VP gene expression is supported by several pieces of evidence. First, the appearance and maximal induction of transcription factors, such as Fos, in parvocellular neurons following an acute challenge occurs in parallel with the induction of VP hnRNA. Second, consensus sequences for AP-1 (Fos/Jun binding site) and NGFI-A and -B response elements have been identified and functionally verified in the regulatory region of the VP gene (GeneBank accession number Y07531) (Chan et al., 1993). Third, protein synthesis inhibitors, which completely prevent the stress-induced synthesis of transcription factors encoded by the IEGs, attenuate VP hnRNA responses to acute stress (Kovács et al., 1998). It should be noted that while c-Fos expression was completely blocked in these studies, VP hnRNA induction was only partially, although significantly, diminished in parvocellular neurons. On the other hand, protein synthesis blockade did not attenuate the rapid rise in the relative levels of CRF primary transcripts, and did not affect the phosphorylation of CREB seen in response to an acute ether challenge in these cells (Fig. 4).

In conclusion, VP transcription is activated within parvocellular neurosecretory neurons in response to acute challenges with a time course that is delayed compared to that of CRF expression and which may involve both phosphorylation events as well as de novo synthesis of transcription factors.

Glucocorticoid feedback on VP gene expression in parvocellular neurosecretory neurons

Compared to the timing of VP gene activation in the parvocellular system, magnocellular VP neurons show an accelerated hnRNA response to an acute osmotic stimulus with a peak rise in hnRNA occurring 30 min following the challenge. This discrepancy further supports that these two neurosecretory systems are distinct populations of vasopressin neurons which utilize different intracellular regulatory mechanisms. In fact, the delay in stress-induced VP gene transcription in the parvo-

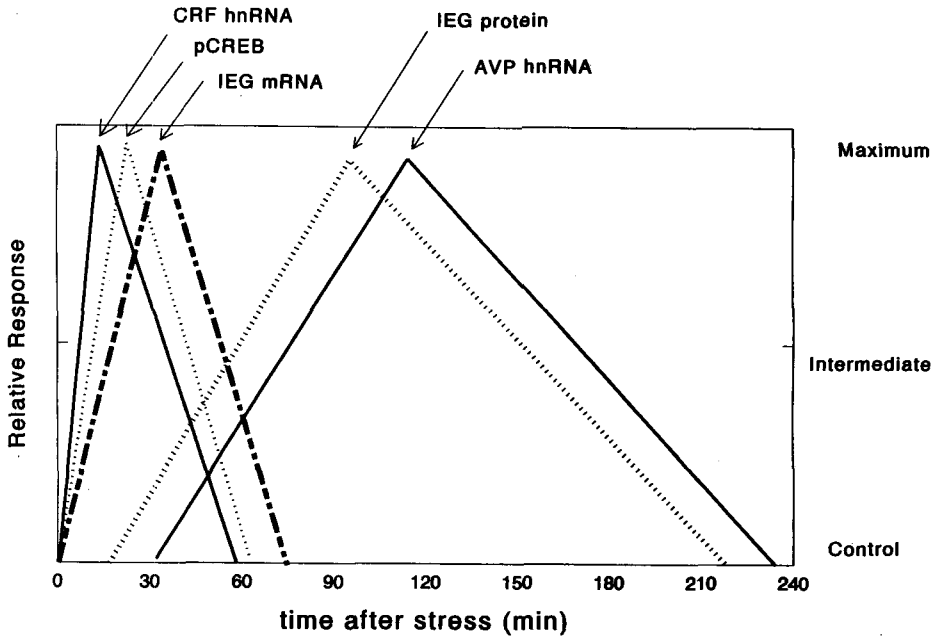

Fig. 4. Sequence of cellular events in the ether stress-induced activation of parvocellular neurosecretory neurons of the PVN. Schematic summary showing the approximate timing of various indices of activation. The early events include a rapid and transient increase of CRF hnRNA and phosphorylation of CREB. Immediate-early genes are activated considerably later, and maximal induction of their protein product is contemporaneous with the maximal stress-induced increases in AVP hnRNA. From Kovács and Sawchenko (1996a).

cellular neurons suggests that additional factors may be involved in the regulation of VP expression in these cells. Our observation that elevations in VP hnRNA in parvocellular neurons are not detected until plasma CORT levels and pCREB are in sharp decline raises the possibility that ligand-activated glucocorticoid receptors arrest VP expression in these cells, as parvocellular neurosecretory neurons are distinguished from magnocellular VP neurons by their capacity to express the type-2 glucocorticoid receptor (Kiss et al., 1988; Uht et al., 1988). Moreover, other evidence suggests that CREB, CREB-phosphorylation, and the CRE itself (Akerblom et al., 1988; Guardiola-Diaz et al., 1996; Légrádi et al., 1997) are targets of glucocorticoid receptor-mediated transcriptional repression. On the other hand, the relatively low levels of CORT required for the inhibition of VP expression under basal conditions suggest that type-1 corticosteroid receptor-mediated mechanisms are also involved (Reul and de Kloet, 1985), and are likely conveyed by afferents from neurons in the limbic system (Herman and Cullinan, 1997).

To identify molecular mechanisms underlying the negative feedback effects of corticosteroids, we compared the timing of ether stress-induced CRF and VP responses in the parvocellular neurons in rats that were either intact, adrenalectomized (ADX), or ADX/CORT-replaced using constant-release steroid pellets to mimic basal AM, basal PM, or peak-stress levels of corticosterone (Dallman et al., 1987). In accordance with previous findings, adrenalectomized rats, in which the endogenous feedback signal has been eliminated, displayed upregulated basal expression of VP mRNA in CRF neurons with no significant changes in magnocellular VP expression (Fig. 5b) (Davis et al., 1986; Herman, 1995). In response to acute ether inhalation, the ADX rats showed increased peak levels of VP primary transcripts over that seen in intact, stressed rats, and in addition, a shift in timing of maximal hnRNA response from 2 h to 5–30 min

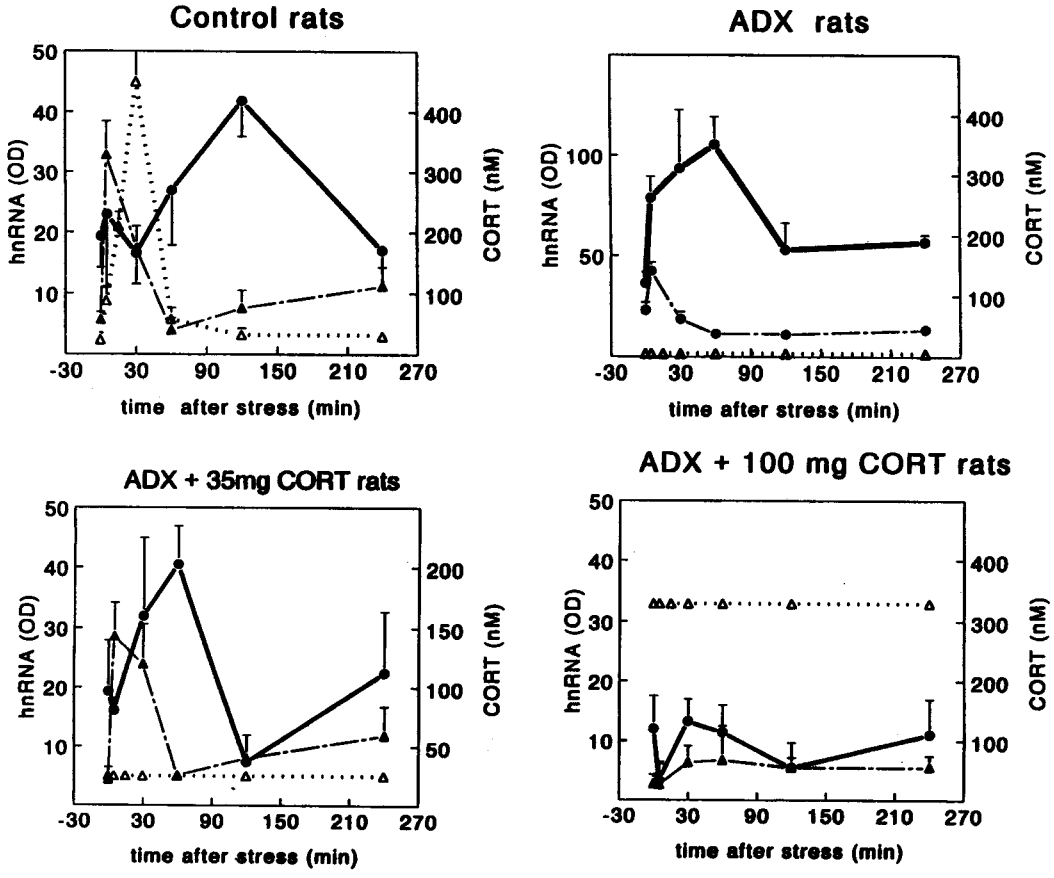

Fig. 5. Time course of ether challenge-induced changes in AVP and CRF gene expression relative to plasma CORT levels. Changes in AVP and CRF hnRNA signals were analyzed in the stress-related parvocellular neurons of intact (a), adrenalectomized (b), adrenalectomized plus CORT-supplemented rats, that were implanted with subcutaneous, constant release pellets to mimic basal morning (c) or peak stress corticosteroid levels (d). Adrenalectomy and CORT replacement was performed 5 days before stress, pellets contained 35 mg CORT resulted constant plasma levels of CORT comparable to basal morning levels, 100 mg CORT pellets provided glucocorticoid levels that are seen at the peak of the acute ether stress. ●---●, AVP hnRNA; ▲---▲, CRF hnRNA; △···△, CORT.

post-stress was observed (Kovács and Sawchenko, 1997). Thereafter, VP primary transcripts decreased and by 2 h post-stress their level was not significantly different from pre-stress values (Fig. 5b). By comparing the areas under the time–response curves for control and adrenalectomized rats, a greater area and thus an enhanced VP response to stress was revealed in the ADX rats compared to controls. The magnitude of challenge-induced VP transcription was normalized in ADX rats that were supplemented with CORT pellets releasing constant, basal levels of corticosterone, but the maximal response was still earlier in these rats compared to intact rats (Fig. 5c). These results strongly suggest that stress-induced CORT plays an important role in delaying the timing of VP gene induction following ether challenge. In fact, supplementing ADX rats with increasing amounts of CORT progressively attenuated basal and stress-induced VP transcription (Herman, 1995; Kovács and Sawchenko, 1997). At the highest levels, ADX rats implanted with a 100-mg CORT pellet (which

releases constant plasma CORT levels comparable to peak levels obtained during stress) did not display any hybridizable VP hnRNA signal in the parvocellular neurons either under basal conditions or after stress (Fig. 5d).

In sharp contrast to this apparent glucocorticoid regulation of stress-induced VP hnRNA responses, the magnitude, timing, and decay of stress-induced CRF transcription was not affected by adrenalectomy or low level glucocorticoid replacement. These results strongly suggest that glucocorticoid feedback during stress is exerted primarily on VP, but not on CRF transcription in the stress-related parvocellular neurosecretory neurons of the hypothalamic PVN. Basal levels of CRF hnRNA were increased in ADX animals consistent with the well-known upregulation of CRF mRNA in the absence of steroid feedback (Kovács et al., 1986; Young et al., 1986b; Swanson and Simmonds, 1989; Herman et al., 1992b), which suggests that a stimulation of transcription rather than altered mRNA stability is responsible for the increased levels of CRF mRNA following ADX. Increased CRF hnRNA levels were normalized in ADX rats implanted subcutaneously with a basal level CORT pellet (Kovács and Sawchenko, 1997). Even higher CORT doses prevented not only ADX-induced, but also stress-induced increases in CRF transcription (Fig. 5a–d).

Transcription factor interactions regulating feedback effect of glucocorticoids on VP expression

The potential mechanisms underlying the feedback regulation of VP (and CRF) transcription may be complex. The elevated hnRNA levels that are seen in ADX rats compared to controls under stress-free conditions suggest an involvement of either tonic, direct, or indirect inhibitory mechanisms in the basal regulation of these neuropeptide genes (Herman and Cullinan, 1997). Upregulation of CRF and VP transcription in ADX rats under basal, stress-free conditions were not accompanied with induction of any of those transcription factors (pCREB, c-Fos or NGFI-B) that have been implicated in the stress-induced activation of CRF or VP genes (Brown and Sawchenko, 1997; Kovács and Sawchenko, 1997). Lack of corticosteroid negative feedback signal in adrenalectomized rats resulted in slight upregulation of CREB phosphorylation, in advanced timing of maximal c-Fos response to acute stress, and increased binding affinity of hypothalamic extracts to AP-1 binding sites and CRE consensus sequences in vitro (Kovács and Sawchenko, 1997). All of these effects might contribute to the exaggerated VP transcriptional response to acute stress. The fact that supplementation of ADX rats with CORT that mimics constant basal, but not the stress-induced peak concentration of CORT, restores the basal and the stress-induced maximal values of VP primary transcripts in the parvocellular neurons, but not the timing of VP hnRNA peak, suggests a 'restraining' role of stress-induced CORT in regulation of vasopressin transcription in vivo. The binding of CORT to its intracellular receptor allows it to bind not only to GRE (glucocorticoid response element), but also to interact with other transcription factors, including CREB and AP-1 complexes, via protein–protein interactions (Diamond et al., 1990; Guardiola-Diaz et al., 1996).

The picture is further complicated by evidence showing that ligand-activated glucocorticoid receptors can both enhance and repress target gene expression through a 'composite' GRE, depending on the presence/absence of the AP-1 components, c-Fos and c-Jun, which serve as selectors of hormone responsiveness (Diamond et al., 1990). The promoter region of the vasopressin gene contains AP-1, GRE, and CRE consensus sequences where such transcription factor interactions could occur (Mohr and Richter, 1990). In addition to these direct hormonal and second messenger-mediated mechanisms, other evidence indicates that there are tonic, negative influences on the parvocellular neurosecretory neurons (Baldino, 1988; Herman et al., 1990, 1992b). Indirect inputs from the limbic system, most likely from the ventral hippocampus, are relayed to local inhibitory GABAergic neurons which might regulate the basal and stress-induced expression of the vasopressin gene (Herman and Cullinan, 1997).

Challenges for the future

Results that are described in this chapter represent our first efforts towards understanding the regulation of the cellular mechanisms underlying neuropeptide gene regulation in vivo. We have initially characterized changes in neuropeptide gene expression as well as the induction of transcription factors in response to an acute stress situation. Similar characterization of transcriptional responses to chronic/repeated stresses would also further our understanding of the intracellular regulatory mechanisms that may be involved in situations more relevant to our everyday life. Moreover, the challenge for the future will be to compare the transcriptional changes that occur during physical and psychogenic stresses. As far as the molecular dissection of vasopressin gene regulation in response to stress, there are many questions remaining to be answered. The increasing availability of new intracellular markers and cell-specific transcription factors will help to identify the mechanisms underlying the relationship between environ-mental stresses and target gene expression in neurosecretory neurons, making this a hot research area in the field of molecular neuroendocrinology.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 1.4

Vasopressin binding sites in the central nervous system: Distribution and regulation

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High affinity binding sites for vasopressin (VP) are widely distributed within the rat brain and spinal cord. Since their presence is associated with neuronal sensitivity to VP application, their anatomical distribution maps structures which could be activated by endogenous VP. Interestingly, marked species-related differences of the VP receptor distribution have been revealed. Some evidence has also been provided that mechanisms of receptor regulation may vary among species. In the rat, the expression of VP binding sites in some motor nuclei shows remarkable plasticity, in particular up-regulation after axotomy. These data suggest that VP may, in addition to affecting motoneuronal excitability, act as a trophic factor onto motoneurones.

Introduction

The pioneering work of De Wied in the 1970s, showing that VP could prevent the extinction of a conditioned avoidance behavior in rats, initiated a large number of studies that further support a role of VP in brain function. In addition to its mnemotonic effects, VP has been implicated in numerous neurobiological functions, which include the central regulation of cardiovascular function and of temperature, the regulation of brain water and electrolyte composition, the control of circadian rhythms, of reproductive behaviors, and of various social behaviors. The effects of VP on centrally regulated functions and behaviors have been summarized and extensively discussed in several recent review articles (Dantzer and Bluthé, 1992; Alescio-Lautier et al., 1993; De Wied et al., 1993).

A major argument supporting the notion that VP exerts central functions has been the discovery of specific high-affinity binding sites in many areas of the rat brain. The pharmacological and binding properties of these sites are similar to those of peripheral V_{1a} receptors. Binding studies have provided no compelling evidence pointing to the presence of either the V_2 or the V_{1b} or other VP receptor subtypes in the central nervous system. The structural and pharmacological properties of central VP receptors compared with those of peripheral receptors have been reviewed recently (Barberis and Tribollet, 1996; Zingg, 1996).

The distribution of central VP binding sites only partially coincides with that of vasopressinergic axon terminals (Sofroniew, 1985). However, recordings of the electrical activity of neurones in areas containing VP binding sites have shown that VP can induce a membrane depolarization and an increase of firing. Thus electrophysiological studies support the notion that binding sites are, at least in part, functional neuronal receptors and that their anatomical localization provides a map of brain structures which can potentially be activated by endogenous VP.

Here we summarize the distribution of central VP binding sites established by light microscopy autoradiography in the adult rat and compare it with distribution patterns observed in other mammalian species. We also report and discuss

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Table 1
Radiolabeled probes for vasopressin receptors

	Dissociation constants (nM) for binding to receptors[a]			
	V_{1a} liver	V_{1b} hypophysis	V_2 kidney	OT (uterus)
[^{3}H]-AVP (a)[b]	3	1–3[d]	0.4[c]	3.5
[^{3}H]-dDAVP (b)	100	51	0.8$^+$	203$^+$
[^{3}H]-d(CH_2)$_5$-[Tyr(Me)^2]AVP (c)	0.3	313	218	–
[^{3}H]-des Gly-NH^{9}d(CH_2)$_5$[D-Tyr(Et)^2,Val^4]AVP (d)	0.2	2040	0.1	–
[^{3}H]-des Gly-NH$_2^9$d(CH_2)$_5$ [D-Ile^2,Ile^4]AVP (e)	–	–	2.8	–
[^{125}I]-d(CH_2)$_5$[Tyr(Me)^2,Tyr-NH$_2^9$]AVP (f)	0.3	–	1	0.13
[^{125}I]-d(CH_2)$_5$[Sar^7]AVP (g)	3.0	–	–	–
[^{125}I]-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (h)	0.06	92	62	1.4
[^{125}I]-Phaa-D-Tyr(Et)-Phe-Gln-Asn-Lys-Pro-Arg-Tyr-NH_2 (i)	0.2	–	–	–
[^{125}I]-HO-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (j)	0.008	4.5	35.5	1.9
[^{125}I]-Nϵ -Tyr-Lys^8-LVP (k)	1.5	–	0.95	–
[^{3}H]-SR 49059 (l)	0.63; 2.9$^+$	220$^+$	275$^+$	1080$^+$
[^{125}I]-d(CH_2)$_5$[D-Tyr(Et)^2, Val^4, Tyr-NH$_2^9$]AVP (m)	0.33	>1000$^+$	1.12$^+$	11$^+$

[a] Dissociation constants reported in this table have been calculated for rat tissues except when noted $^+$ where they have been calculated for human receptors.

[b] References: (a) Cantau et al. (1980); Lutz-Bucher and Koch (1983); Antoni (1984); Koch and Lutz-Bucher (1985); Audigier and Barberis (1985). (b) Cantau et al. (1980); Lolait et al. (1995); Marchingo et al. (1988); Chini et al. (1995). (c) Dorsa et al. (1988); Antoni (1984); (d) Stassen et al. (1987); Jard et al. (1986). (e) Trinder et al. (1991). (f) Elands et al. (1988). (g) Kelly et al. (1989); Gerstberger and Fahrenholz (1989). (h) Schmidt et al. (1991). (i) Strakova et al. (1997). (j) Barberis et al. (1995). (k) Durr et al. (1992). (l) Serradeil-Le Gal et al. (1993, 1994). (m) Ala et al. (1997).

examples of regulation of receptor expression, in particular variations observed in the rat somatic motor nuclei which suggest that VP may act as a neurotrophic factor.

Radiolabelled ligands

Several radiolabelled ligands are available to study VP receptors. Their binding characteristics are given in Table 1. Early autoradiographic investigations on brain sections were performed with tritiated VP which binds to all subtypes of VP receptors and to oxytocin (OT) receptors with similar affinities. Competition studies using non-radioactive selective analogues together with tritiated VP showed that only V_{1a}-like VP receptors were detectable within the brain. Subsequent studies conducted with more selective radiolabelled compounds yielded similar conclusions.

Among V_{1a} selective radiolabelled ligands, the linear VP antagonist HO-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (Manning et al., 1992) monoiodinated in position 1 (^{125}I-VPA) has proved to be particularly suitable for light microscopy in vitro autoradiography (Barberis et al., 1995). Its high affinity and selectivity combined with high specific activity and low non-specific binding make it the most sensitive radioligand available so far for central V_{1a} VP receptors (see Table 1).

Several imaging systems allowing the fast and accurate detection of low energy emitting isotopes have been recently developed. In that respect, the tritiated form of the new potent and non-peptide VP antagonist SR49059 is of interest. It has a good affinity for both rat and human V_{1a} receptors (Serradeil-Le Gal et al., 1993, 1994) and has already been used successfully for the autoradiographic detection of V_{1a} receptors in the rat kidney (Serradeil-Le Gal et al., 1996) and brain (Tribollet et al., 1998).

Table 2
Distribution of vasopressin and oxytocin binding sites in the rat brain[a]

	Vasopressin binding sites
Cerebral cortex	
Neocortex	
Layer (V	(+)
Layer VI	(+)
Pyriform cortex	+
Olfactory system	
Olfactory bulb	
Internal plexiform layer	(+)
External plexiform layer	(+)
Ependymal layer	(+)
Anterior olfactory nucleus	++
Basal ganglia	
Accumbens nucleus	++
Fundus striati	+++
Nucleus of the diagonal band	(+)
Limbic system	
Lateral septal nucleus	+++
Bed nucleus of the stria terminalis	++
Amygdalostriatal transition area	++
Field CA1 of Ammon's horn	+
Field CA2 of Ammon's horn	(+)
Field CA3 of Ammon's horn	+
Dentate gyrus	++
Thalamus	
Anteroventral nucleus	+
Reticular nucleus	(+)
Ventromedial nucleus	+
Ventrolateral nucleus	+
Paraventricular nucleus	+
Mediodorsal nucleus	(+)
Posterior nucleus	(+)
Subparafascicular nucleus	(+)
Hypothalamus	
Magnocellular preoptic nucleus	(+)
Suprachiasmatic nucleus	++
Lateral hypothalamic area	++
Arcuate nucleus	++
Perifornical nucleus	+
Zona incerta	++
Stigmoid nucleus	+++
Tuber cinereum	++
Midbrain	
Mesencephalic central gray	(+)
Rostral linear raphe nucleus	++
Dorsal raphe nucleus	++
Substantia nigra	+
Interpeduncular nucleus	++
Nucleus of Darkschewitsch	++
Superior colliculus	++
Ventral tegmental area	+
Edinger-Westphal nucleus	++
Interstitial nucleus of the medial longitudinal fasciculus	(+)
Medulla-pons	
Locus coeruleus	++
Parvocellular reticular nucleus	+
Nucleus of the solitary tract	+++
Hypoglossal nucleus	(+)
Inferior olive	+++
Spinal trigeminal nucleus, interpolar part	+
Spinal cord	
Laminae 1-2	+
Laminae 3-6	+
Laminae 7	+
Laminae 8	+
Laminae 9	+
L8–Th1 dorsolateral motor nucleus	++
L2–L5 medial motor nucleus	++
L5–L6 retrodorsolateral nucleus	+++
L5–L6 pudendal nuclei	+++
Circumventricular organs	
Subfornical organ	(+)
Pineal gland	+
Area postrema	+++

Table 2 (*continued*)

	Vasopressin binding sites
Choroid plexus	+++
Superficial arteries	+++

[a] All regions found specifically labelled with ^{125}I-VPA are reported (Barberis et al., 1995; Tribollet et al., 1997). (+), just detectable; +, low density; ++, moderate density; +++, high density.

Distribution and regulation in the adult rat

High affinity binding sites specific for VP are widely distributed within the rat central nervous system. To a few exceptions, the map of VP binding sites does not overlap that of OT binding sites (Tribollet, 1992). The existence of different receptor binding patterns for the two peptides supports the view that centrally released VP and OT subserve distinct physiological functions by activating different neuronal pathways. Evidence has been provided on the neuronal networks likely responsible for central effects of VP by mapping the expression of c-fos after intracerebroventricular injection of VP in rats (Giri et al., 1990; Andrae and Herberts, 1993).

Table 2 gives an exhaustive list of the rat brain regions found labelled with the sensitive V_{1a} receptor ligand, ^{125}I-VPA (see Table 1). It includes brain areas described previously with other radiolabelled ligands (reviewed by Tribollet, 1992; Barberis and Tribollet, 1996) and several additional areas containing low densities of binding sites revealed only by ^{125}I-VPA (Barberis et al., 1995).

Recent data show that the spinal cord is rich in VP binding sites (Tribollet et al., 1997). A moderate and diffuse labelling of the central gray matter was observed at all spinal levels with ^{125}I-VPA. In addition, high concentrations of VP binding sites were found in a few groups of somatic motoneurones, in particular in the pudendal motor nuclei which innervate the sexually dimorphic striated perineal muscles (Fig. 1).

Labelling of the superficial arteries supplying the brain is noteworthy since it suggests that VP may play a role in the regulation of cerebral blood flow.

Central VP receptors have not yet been cloned, but the expression of VP receptors mRNAs has been investigated in the rat brain by using probes constructed from known sequences of peripheral receptors. The notion that central VP binding sites correspond to peripheral V_{1a} receptors has been supported recently by in situ hybridization data showing a distribution of V_{1a} receptor mRNA consistent with that of binding sites (Ostrowski et al., 1994). The data were obtained by using a riboprobe containing the sequence coding for the third putative transmembrane loop of the V_{1a} receptor. Surprisingly however, a strikingly different distribution has been established with a probe directed against the untranslated region of the V_{1a} receptor mRNA (Szot et al., 1994).

There is no indication so far for the presence of V_2 receptor mRNA in the adult rat brain (Ostrowski et al., 1992; Saito et al., 1995), while it may be expressed transiently during development (Saito et al., 1995). V_{1b} receptor mRNA has been evidenced by reverse transcription polymerase chain reaction only (Hirasawa et al., 1994; Lolait et al., 1995), which signifies a low amount of expression probably accounting for the undetectability of V_{1b} binding sites by autoradiography.

Electrophysiological recordings, done mostly in brain slices, have shown a good correlation between the presence of VP binding sites and the neuronal responsiveness to VP. It was shown by intra-cellular recordings that the effect of VP is direct, postsynaptic, meaning that at least part of the VP binding sites detected by autoradiography are located on neurones (see De Kloet et al., 1990 and Raggenbass et al., this volume). This does not exclude however, the possibility that VP binding sites are also present on non-neuronal cells, i.e. glial or endothelial cells (Jurzak et al., 1995; Yamazaki et al., 1997).

Mechanisms of VP receptor regulation in the rat central nervous system have not been much investigated. In sharp contrast with OT binding sites which are markedly dependant upon circulating gonadal steroids, the amount of VP binding sites detectable in the brain is not modified by castration

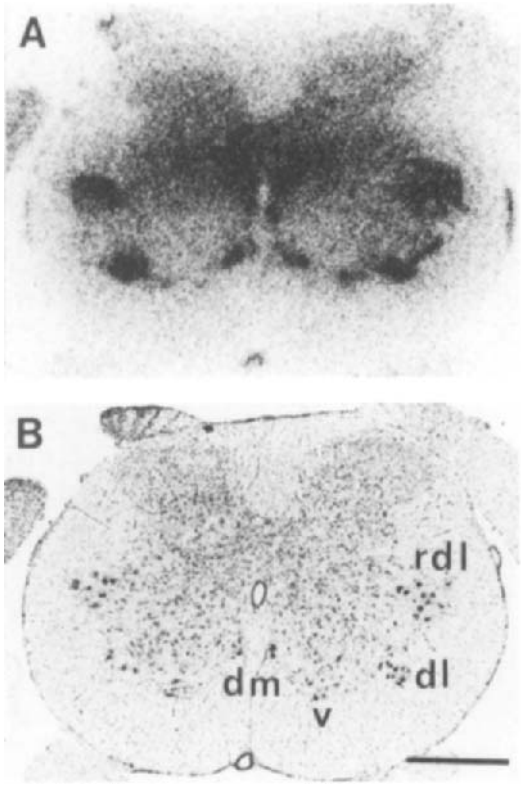

Fig. 1. Localization of AVP receptors in segment L6 of the male rat brain. (A) Autoradiogram obtained from section shown in (B) incubated with ^{125}I-VPA. (B) Photograph of the section stained with cresyl-violet. Note the diffuse labelling of central gray and the intense labelling of motor nuclei. dl, dorsolateral nucleus; dm, dorsomedial nucleus; rdl, retrodorsolateral nucleus; v, ventral nucleus. Bar = 1 mm.

or by hormonal treatment (Tribollet et al., 1990). However, castrated male rats show a decreased level of VP receptor binding in lumbo-sacral spinal motor nuclei (Tribollet et al., 1997). Adrenalectomy has been reported to induce a significant decrease of tritiated VP binding on hippocampal membranes (Saito et al., 1994) and of V_{1a} mRNA in septum and bed nucleus of the stria terminalis (Watters et al., 1996).

Distribution and regulation in other mammalian species

Mapping studies carried out in several mammals including humans have revealed marked species-related differences. The distribution of VP binding sites have been investigated in the rat (see above), the mouse (Insel et al., 1991; Dubois-Dauphin et al., 1996), the guinea-pig (Tribollet et al., 1992a), the golden and Siberian hamsters (Dubois-Dauphin et al., 1990, 1991, 1994), prairie and montane voles (Insel et al., 1994; Young et al., 1997), the jerboa (Théler et al., 1993), the Mongolian gerbil (Vallet et al., 1995), the marmoset (Tribollet et al., 1992b; Wang et al., 1997), and the human (Loup et al., 1991). The main features of species-related differences observed can be summarized as follows.

First, only a few areas show comparable high amounts of VP receptor binding in all species studied, namely the septal area, the area postrema and the nucleus of the solitary tract. Second, in many regions, the amount of VP binding sites varies tremendously between species. For instance, the hippocampus is not labelled in the guinea-pig and in the Mongolian gerbil, intensely labelled in the jerboa, and only moderately labelled in all the other species studied. Similar variations have been shown in the cerebral cortex where the density of VP binding sites ranges from just detectable in the rat or mouse to very high in the Mongolian gerbil. Third, a most intriguing observation is that a few areas appear to contain high amounts of either VP or OT binding sites depending upon the species. For instance, the hypothalamic ventromedial nucleus contains OT binding sites in the rat, mouse, and guinea pig, but VP binding sites in the hamster and marmoset (rat and marmoset are illustrated in Fig. 2). The hypothalamic ventromedial nucleus subserves different functions in different species. An alternative interpretation may be that depending upon the species, the activity of hypothalamic ventromedial neurones is regulated by either OT or VP. Remember that receptors are not entirely specific for VP or OT.

Further studies in the hamster have demonstrated that VP binding sites are regulated by circulating gonadal steroid hormones in the hypothalamic ventromedial nucleus, but not in other structures. They are more numerous in males than in females (Delville and Ferris, 1995), reduced by castration in males (Dubois-Dauphin et al., 1994; Johnson et al., 1995; Delville et al., 1996), and they disappear in

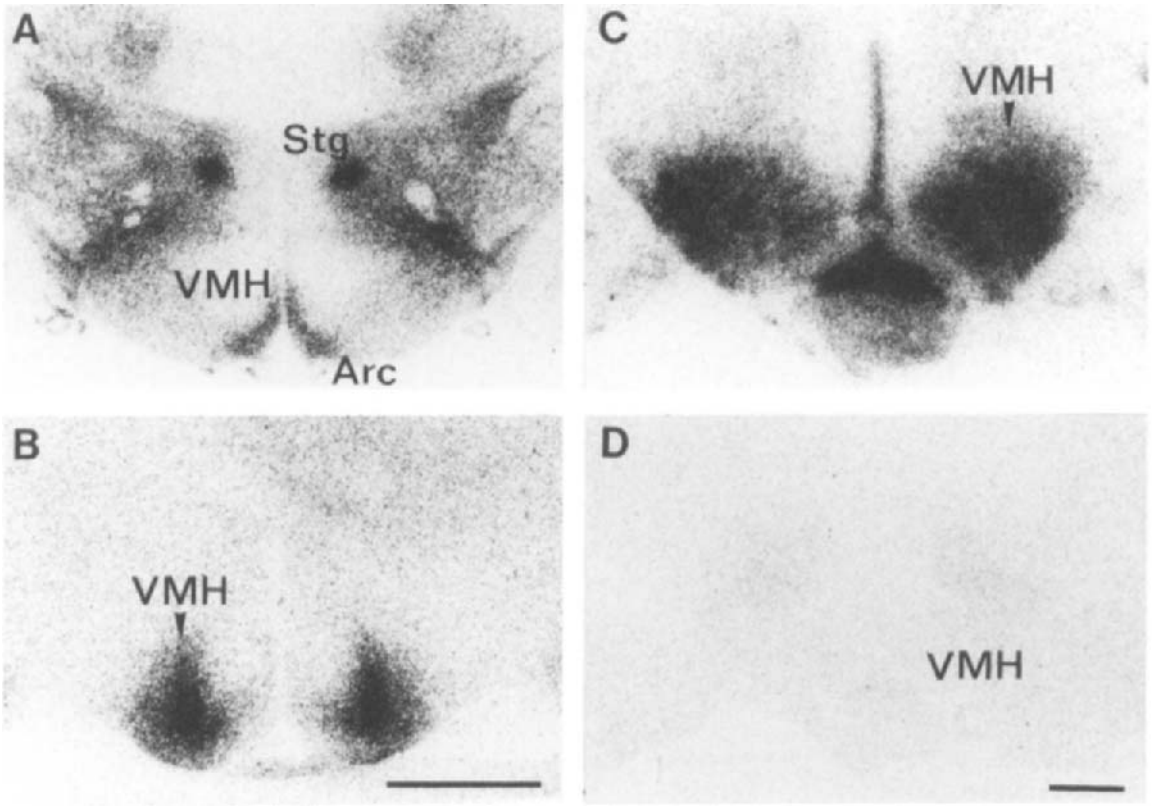

Fig. 2. Autoradiograms showing AVP and oxytocin binding sites in the hypothalamus of the rat (A,B) and marmoset (C,D). (A,C) show labelling yielded by the selective V_{1a} antagonist ^{125}I-VPA, and (B,D) the labelling obtained in adjacent sections with ^{125}I-OTA, a ligand selective for oxytocin receptors (Elands et al., 1988). Note that in the rat, the hypothalamic ventromedial nucleus (VMH) contains oxytocin binding sites and no AVP binding sites. In contrast, only AVP binding sites are detectable in the VMH of the marmoset. Other abbreviations: Arc, hypothalamic arcute nucleus; Stg, stigmoid hypothalamic nucleus. Bars = 1 mm.

lactating females (Delville et al., 1995). Interestingly, OT hypothalamic ventromedial receptors are controlled by gonadal hormones in the rat, but not in the guinea pig (review in Barberis and Tribollet, 1996). These observations and others indicate that the mechanisms of VP receptor regulation vary among species, in particular the dependency upon gonadal steroid hormones, and that, in addition, these mechanisms are region specific for a given species.

One tempting explanation for the extensive species-related differences in VP and OT receptor topography is that these receptors mediate species-typical behaviors. This hypothesis is supported by comparative studies demonstrating markedly different patterns of VP and OT receptor distribution in species that are similar in most aspects but differ by their monogamous versus polygamous behavior (Insel et al., 1994; Young et al., 1997). In one of these monogamous species, central VP indeed facilitated the development of partner preference in males (Winslow et al., 1993).

Plasticity of expression in the rat somatic motor nuclei

The expression of VP receptors in the rat somatic motor nuclei shows remarkable changes both

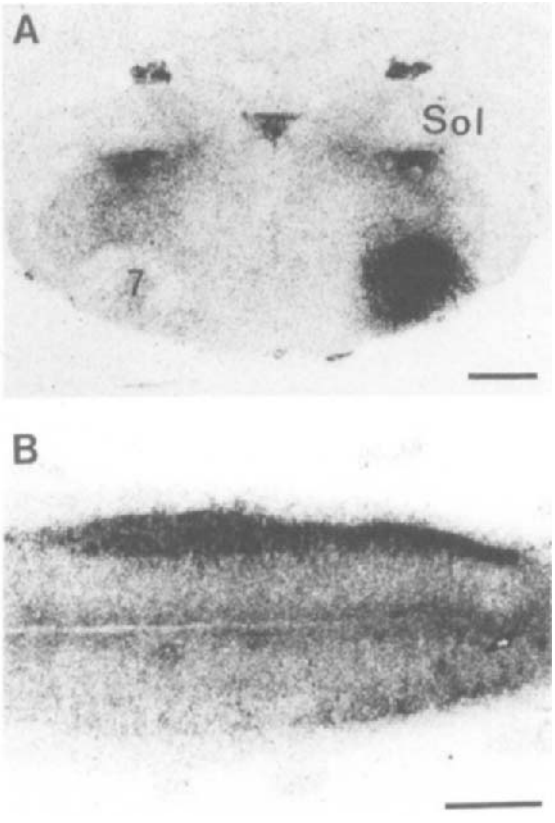

Fig. 3. Axotomy-induced AVP binding sites in the facial nucleus (A) and sciatic motor nuclei (B) 3 weeks following section of the right facial nerve and the right sciatic nerve, respectively. Autoradiograms were obtained from tissue sections incubated with the selective V_{1A} receptor ligand, ^{125}I-VPA (Barberis et al., 1995). (A) is from a coronal brainstem section; (B) is from a longitudinal section of the spinal cord at the level of lumbar enlargement. Right is on the top, left on bottom. 7, facial nucleus; Sol, nucleus of the solitary tract. Bars = 1 mm.

during development and following axotomy. Thus, the facial nucleus which innervates the superficial muscles of the head and neck, is the structure containing the highest number of VP binding sites during the late embryonic and early postnatal period; they are undetectable by autoradiography in the adult (Tribollet et al., 1991). However, section of the facial nerve in the adult rat induces a massive and long lasting re-expression in the deafferented nucleus, reaching a maximal level 1–2 weeks following axotomy (Tribollet et al., 1994) (Fig. 3A). A similar strong expression of VP binding sites is observed in the hypoglossal nucleus and in sciatic motor nuclei following section of the corresponding nerves (Tribollet et al., 1994) (Fig. 3B). The pharmacological profile of facial binding sites expressed during development or following axotomy in adulthood is indistinguishable from that of peripheral V_{1a} receptors.

Results of developmental studies support the hypothesis that VP receptors are involved in the establishment of neuromuscular connections. Although synaptic contacts between motoneurones and skeletal muscle cells are formed during embryonic life, the adult pattern of mononeuronal innervation of muscle fibers is achieved only during the critical postnatal period (for a review, see Jansen and Fladby, 1990). VP receptors are expressed transiently by facial motoneurones during this period characterized by the reorganization of neuromuscular connections.

The response of motoneurones to axotomy has been extensively studied (Aldskogius and Svensson, 1993). In general terms, changes induced can be interpreted as an increased expression of compounds that promote survival and axonal regrowth, and a decrease of those related to neurochemical transmission. Accordingly, receptors for trophic factors are up-regulated in contrast to receptors for neurotransmitters which are down-regulated. In that context, it is noticeable that the expression of VP receptors does not follow that of receptors for neurotransmitters, but is increased similarly to receptors for trophic factors. This suggests that VP may take part in regenerative mechanisms leading to restoration of connections between the lesioned motoneurones and their target muscles.

Interestingly, at variance with cranial motor nuclei and most spinal ones, a few groups of spinal motoneurones express VP receptors permanently (Tribollet et al., 1997). Pudendal nuclei in particular, are strongly labelled with ^{125}I-VPA in adult rats, the labelling pattern showing the expected sexually dimorphic pattern (see Fig. 1 for labelling in the male). Actually, pudendal motoneurones differ from most other somatic motoneurones by showing permanently a number of features normally observed only during development or following

axotomy (for a review, see Tribollet et al., 1997). It is also of interest that castration reduces ^{125}I-VPA binding in pudendal nuclei of the male rat. So far, this is the first evidence that a variation of circulating gonadal steroids may influence VP receptor gene expression in the rat central nervous system.

The possibility that VP could act as a neurotrophic factor has not been investigated extensively. It was first suggested by studies on the VP-deficient Brattleboro rat which exhibit retarded brain growth and a reduced number of cerebellar granule cells in adulthood (Boer, 1985). VP has been shown to promote neurite outgrowth of cultured embryonic neurones from *Xenopus laevis* (Brinton and Gruener, 1987), rat hippocampus (Clos and Gabrion, 1989; Brinton et al., 1994) and rat ventral spinal cord (Ikeda et al., 1989) in vitro. In contrast to the paucity of information related to trophic effects of VP in the central nervous system, a large body of evidence has been provided on growth-promoting and/or mitogenic effects in a variety of non-neuronal cell types (for a review, see Carter et al., 1993). In many instances, these effects have been shown to be mediated via activation of V_{1a} receptors.

Concluding remarks

Our knowledge about the central vasopressinergic system has much increased during the last decade. Receptors specific for VP have been identified within numerous areas of the brain and spinal cord in the rat and other mammals, although with marked species-related differences in their distribution. The functional relevance of these receptors has been demonstrated at the cellular level by electrophysiological studies showing that VP can induce neuronal depolarization. On the other hand, evidence has been produced that VP may influence many centrally regulated functions. However, we are still lacking an integrative view of the variety of VP effects described and of their physiological significance.

The induction of VP receptor expression by axotomy in somatic motor nuclei and their disappearance following muscle reinnervation suggests that VP may, in addition to its well demonstrated short-term effects at the membrane level, exert long-term changes in motoneurones, possibly by regulating the expression of genes promoting motoneuronal survival and regeneration. Motoneurones and their connections with skeletal muscle cells provide a good model to investigate the putative neurotrophic function of VP as well as the mechanisms of VP receptor gene expression.

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SECTION 2

Cellular properties of vasopressinergic neurons

Electrophysiological properties
Cell and molecular biology of vasopressin neurons

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CHAPTER 2.1.1

Osmoregulation of vasopressin neurons: A synergy of intrinsic and synaptic processes

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The release of vasopressin into the general circulation varies as a function of plasma osmolality and therefore plays a major role in systemic osmoregulation. In vivo, the secretion of this hormone in the neurohypophysis is primarily determined by the rate of action potential discharge of the magnocellular neurosecretory cells (MNCs) in the hypothalamus. Experiments done over the past 20 years have clarified much of the neurophysiological basis underlying this important osmoregulatory reflex. As discussed here, recent findings indicate that the regulation of the firing rate of MNCs during changes in systemic osmolality involves the concerted modulation of mechanosensitive ion channels in MNCs, as well as excitatory glutamatergic inputs derived from forebrain regions such as the organum vasculosum of the lamina terminalis.

Introduction

In vertebrates, excessive gain or loss of free water through changes in intake, evaporation or excretion, can provoke perturbations in intracellular and extracellular solute concentrations that can have serious pathological consequences (Arieff and Guisado, 1976; Arieff et al., 1976; Arieff, 1986). One of the main osmoregulatory responses opposing changes in systemic solute concentrations involves the antidiuretic hormone vasopressin (VP). It has long been known that the release of VP from the neurohypophysis increases as a function of plasma osmolality (P_{OSM}; e.g., Verney, 1947), thereby ensuring that the amount of water being reabsorbed by the kidney is proportional to the body's need for free water. The osmotic control of VP release, therefore, plays a key role in body fluid and cardiovascular homeostasis. Here, the neurophysiological basis for the osmotic regulation of VP-secreting neurosecretory neurons is examined in the light of recent findings concerning the involvement of mechanisms intrinsic to the VP cell, and of extrinsic synaptic influences.

Osmotic control of vasopressin secretion

Osmotic set-point

In mammals, P_{OSM} is generally maintained near a constant 'set-point'. Under resting conditions, the value of the set-point is similar for different individuals in a given species, but may vary from one species to the next. For example, resting P_{OSM} is about 287 mosmol/kg in healthy humans (e.g., Robertson et al., 1976), 294 mosmol/kg in rats (e.g., Dunn et al., 1973) and 320 mosmol/kg in cats (e.g., Reaves et al., 1981). Deviations of P_{OSM} from the osmotic set-point are among the most potent stimuli regulating VP release in vivo (Verney, 1947). Increases in P_{OSM} as small as +1% (i.e., about +3 mosmol/kg) are sufficient to provoke measurable increases in the concentration of VP in plasma (e.g., Dunn et al., 1973; Schrier et al., 1979) and to enhance water reabsorption in the kidney (Robertson et al., 1976). Previous studies in a variety of species have shown that under resting osmotic conditions (i.e., when P_{OSM} = set-point), the concentration of vasopressin in plasma is about 2–3 pg/ml (for review, see Bie, 1980), a value that is near the middle of the dose-response range for the

renal concentrating mechanism (Robertson et al., 1976). Consequently, decreases of the concentration of VP in plasma resulting from systemic hypotonicity (i.e., when P_{OSM} falls below set-point) essentially stimulate diuresis. The presence of an active basal hormone concentration at the osmotic set-point, therefore, is a functionally important feature for systemic osmoregulation (Bourque et al., 1994).

Osmotic threshold

Under conditions where the value of P_{OSM} decreases progressively below the set-point the concentration of VP in blood can ultimately fall below ~0.3 pg/ml, the lowest value that can promote a measurable antidiuresis (Robertson et al., 1973; Robertson et al., 1976). This observation suggests that there is an apparent functional osmotic 'threshold' for VP secretion when P_{OSM} increases from an initially hypotonic value. For individual species the value of this apparent threshold, relative to the osmotic set-point, depends on the experimental protocol used to generate the data, and on whether linear-linear (e.g., Dunn et al., 1973; Robertson et al., 1976; Koehler et al., 1993) or log-linear (Weitzman and Fisher, 1977; Weisinger et al., 1993) regression analysis is used to fit the VP–P_{OSM} relationship. In the rat, linear regression analysis of data taken during systemic osmotic stimulation suggests that VP levels fall to zero about 4 mosmol/kg below set-point (i.e., near 290 mosmol/kg; e.g., Dunn et al., 1973; Koehler et al., 1993). Linear regressions, however, tend to overestimate the slope of the relationship below set-point, as indicated by Robertson (1985), and typically overlook the low but functional levels of plasma VP that can be measured at osmolalities below the x-intercept. This type of analysis, therefore, underestimates the functional osmotic threshold for VP release. Log-linear regression analysis, which takes into consideration the fact that the slope of the VP–P_{OSM} relation tends to increase with P_{OSM} (Weitzman and Fisher, 1977), indicates that in rats the osmolality at which VP levels first become detectable by radioimmunoassay (about 0.3 pg/ml) lies near 282 mosmol/kg (Weisinger et al., 1993). Such analysis provides a better fit of the data points obtained at the lower range of P_{OSM} values and may therefore better reflect the relationship between plasma VP concentration and P_{OSM} near the physiological set-point. Taking into consideration the fact that the renal concentrating mechanism begins to respond to plasma VP at concentrations near 0.3 pg/ml (Robertson et al., 1973), a value of 282 mosmol/kg may be taken to represent the functional osmotic threshold for VP secretion. The plot shown in Fig. 1A illustrates, in linear-linear form, the general features of the VP–P_{OSM} relation obtained by log-linear analysis (Weisinger et al., 1993) in control rats.

The VP–P_{OSM} relationship reflects the firing rate of VP neurons

Following its synthesis in the somata of magnocellular neurosecretory cells (MNCs) in the hypothalamic supraoptic and paraventricular nuclei, VP is packaged into secretory vesicles and is shipped by fast axonal transport to axon terminals in the neurohypophysis (Brownstein et al., 1980) where release occurs in a Ca^{2+}-dependent manner (e.g., Douglas, 1963; Mikiten and Douglas, 1965). The secretion of VP in the neurohypophysis is triggered by the propagation of tetrodotoxin-sensitive action potentials into the nerve terminals (Dutton and Dyball, 1979) and presumably occurs following activation of local voltage-gated Ca^{2+} channels (Lemos and Nowycky, 1989; Bookman et al., 1991; Fisher and Bourque, 1995a, 1996), intracellular Ca^{2+} accumulation (Brethes et al., 1987; Jackson et al., 1991) and exocytosis (Fidler Lim et al., 1990). The axon terminals of MNCs cannot fire repetitively in response to sustained injection of depolarizing current, but can be excited at high frequencies by repetitive stimulation of the axon with brief current pulses (Bourque, 1990). The control of VP secretion in situ, therefore, is achieved through a regulation of the rate and pattern of discharge of action potentials initiated at the MNC somata (Poulain and Wakerley, 1982; Bourque and Renaud, 1990).

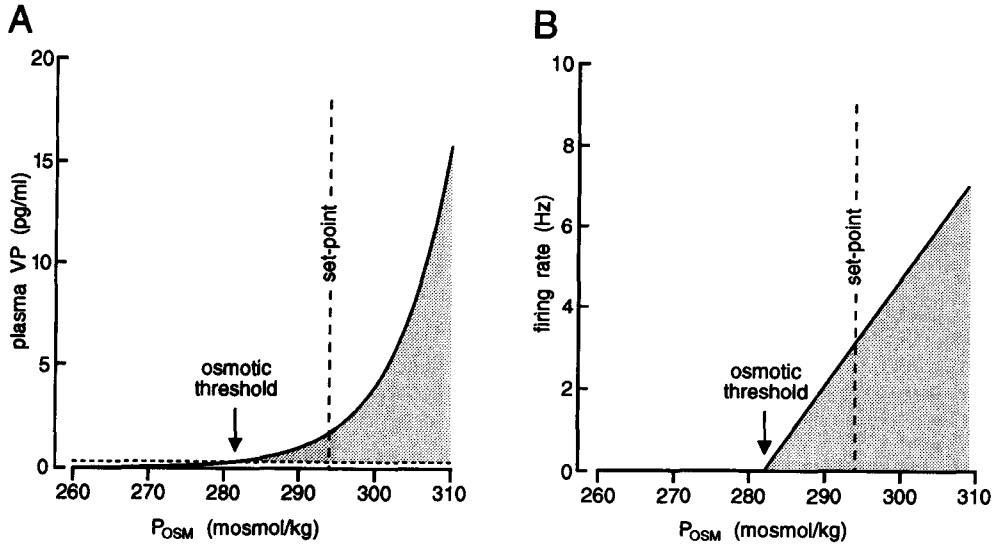

Fig. 1. Effects of P_{OSM} on VP release and firing rate in MNCs. The solid line in (A) plots, on a linear-linear scale, the relationship between plasma VP concentration and P_{OSM}, derived from the log-linear fit of data obtained in control rats by Weisinger et al. (1993). The line plots $y = 10^{(0.06x - 17.4)}$, where $x = P_{OSM}$. The dashed horizontal line shows where VP concentration is 0.3 pg/ml, which is used here to define the functional osmotic threshold (arrow) for VP release. Note that a plasma VP concentration of ~2 pg/ml prevails at the osmotic set-point (vertical dashed line). The plot in (B) shows the average relationship between mean overall firing rate and P_{OSM} in MNCs. The slope of the plot (0.25 Hz/mosmol kg^{-1}) represents the mean of the slopes of linear regression fits reported from experiments in anaesthetized rats (Walters and Hatton, 1974; Brimble and Dyball, 1977; Wakerley et al., 1978; see text for details). The line draws $Y = 0.25x - 70.5$ (>282 mosmol/kg) and $y = 0$ (≤282 mosmol/kg); the y-offset of the line was adjusted to yield a frequency of 3 Hz (the mean basal firing rate of MNCs under resting osmotic conditions) at the osmotic set-point.

Relation between P_{OSM} and firing rate

Extracellular recordings from anaesthetized rats have revealed that under conditions where P_{OSM} lies near the osmotic set-point (i.e., near 294 mosmol/kg) VP-MNCs discharge action potentials at a mean rate of about 3 Hz (e.g., Walters and Hatton, 1974; Brimble and Dyball, 1977; Poulain et al., 1977; Wakerley et al., 1978), a frequency sufficient to provoke the release of VP from the isolated rat neurohypophysis in vitro (e.g., Dutton and Dyball, 1979), and which explains the presence of a significant basal plasma VP concentration at rest (e.g., Dunn et al., 1973). When P_{OSM} is reduced by an intragastric water load, the firing rate of VP-MNCs decreases (Brimble and Dyball, 1977). Conversely, increases in P_{OSM} evoked by i.p. injection of hypertonic saline (Brimble and Dyball, 1977), or by progressive dehydration (Walters and Hatton, 1974; Wakerley et al., 1978) provoke an increase in mean firing rate. Although such an analysis is complicated by the fact that hyperosmotic stimulation often provokes or modulates phasic firing in VP-MNCs (consecutive on and off periods of activity lasting tens of seconds each), linear regression analysis performed in the studies by Brimble and Dyball (1977) and Wakerley et al. (1978) confirmed that mean firing rate and P_{OSM} were correlated, with slopes of 0.42 ($R = 0.65$) and 0.087 Hz/mosmol kg^{-1} ($R = 0.59$), respectively. Consistent with these results, post-hoc linear regression analysis of the relation between intraburst firing rate in phasically active (putative VP-releasing) MNCs (data taken from Fig. 2 in Walters and Hatton, 1974), and corresponding P_{OSM} (Table 2 in the same paper) revealed a slope of 0.24 Hz/mosmol kg^{-1}. The variance in the firing rate versus P_{OSM} data taken from different cells (Brimble and Dyball, 1977; Wakerley et al., 1978) and the recruitment

of various forms of patterned activity during hypertonic stimulation (Walters and Hatton, 1974; Brimble and Dyball, 1977; Poulain et al., 1977; Wakerley et al., 1978; Bourque and Renaud, 1984) make it difficult to evaluate the suitability of analyzing the relation between mean firing frequency and P_{OSM} using linear regression. Based on the information available, however, the apparent osmotic threshold for eliciting action potential firing in VP-MNCs in vivo (Fig. 1B) is in good agreement with that characterizing the VP–P_{OSM} relation observed using radioimmunoassay (Fig. 1A). The apparent nonlinear relation between firing rate and secretion at values near the osmotic set-point (Fig. 1A,B) is not unexpected given that, over the range of firing rates concerned, VP release per action potential is enhanced in a frequency-dependent manner (Bicknell, 1988) and is further enhanced by the appearance of phasic firing (Dutton and Dyball, 1979; Bicknell and Leng, 1981).

Osmotic control of firing rate in VP neurons

The frequency and pattern of spike discharge in MNCs is known to arise as a result of interactions between intrinsic membrane properties and afferent synaptic influences (e.g., Bourque and Renaud, 1990; Bourque et al., 1998). In agreement with this principle, the osmotic regulation of VP secretion into blood reflects the integrated effects of both endogenous processes and synaptic influences on the firing rate and pattern of VP-MNCs (Bourque et al., 1994). Although it has been known for more than 50 years that changes in P_{OSM} regulate VP release via centrally located osmoreceptors (Verney, 1947), details of the cellular and synaptic processes mediating the osmotic control of action potential discharge in MNCs only began to emerge with the introduction of in vitro methods permitting sharp electrode or whole cell patch-clamp recording of membrane current or voltage.

Resting potential in MNCs is subthreshold at the osmotic set-point

At osmolalities near the osmotic set-point the resting membrane potential of MNCs measured during sharp electrode recordings is, on average, near −63 mV (e.g., −67 mV (Mason, 1983); −66 mV (Bourque et al., 1986); −68 mV (Randle et al., 1986); −64 mV (Gribkoff and Dudek, 1990); −59 mV (Hu and Bourque, 1991); −64 mV (Armstrong et al., 1994); −58 mV (Inenaga et al., 1994); −60 mV (Yang and Hatton, 1994)). Action potential threshold, though more variable because of differences in measurement criteria, is generally observed to be near−50 mV (e.g., −55 mV (Mason, 1983); −55 mV (Richard and Bourque, 1995); −44 mV (Stern and Armstrong, 1996);−50 mV (Ghamari-Langroudi and Bourque, 1998)). Therefore the presence of basal electrical activity at rest in MNCs is dependent on the occurrence of transient depolarizing influences such as excitatory postsynaptic potentials (EPSPs; e.g., Mason, 1983; Gribkoff and Dudek, 1990; Wuarin and Dudek, 1993; Richard and Bourque, 1995; Inenaga et al., 1997), non-synaptic depolarizing potentials (Bourque et al., 1986), post-spike depolarizing after potentials (DAPs) (Andrew and Dudek, 1983; Andrew and Dudek, 1984), or membrane currents promoting rebound excitation (Erickson et al., 1993; Fisher and Bourque, 1995b; Stern and Armstrong, 1996). Since a large proportion of the depolarizing transients observed under resting conditions are too small to provoke firing (e.g., Mason, 1980, 1983; Bourque et al., 1986), electrical excitation in response to a hypertonic stimulus could potentially arise from an increase in the frequency or amplitude of the depolarizing transients and/or from membrane depolarization, which would effectively increase the proportion of depolarizing transients capable of eliciting action potentials.

MNCs are intrinsically depolarized by hypertonic stimuli

It was Verney (1947) who first speculated that the cerebral osmoreceptors regulating VP release might reside directly within the supraoptic nucleus. In agreement with this hypothesis, extracellular recordings in anaesthetized rats have revealed that localized application of hypertonic saline in the immediate vicinity of hypothalamic MNCs results in electrical excitation (Leng, 1980). Similarly, local infusion of strongly hypertonic saline into

hypothalamic nuclei containing MNCs has been found to provoke systemic VP release (Ludwig et al., 1994, 1995). Conclusive evidence that MNCs are intrinsically osmosensitive, however, was first provided by Mason (1980), who showed that MNCs in hypothalamic slices in vitro are depolarized when superfused with hypertonic solutions, and that this response persists when chemical synaptic transmission is blocked by low Ca^{2+}/high Mg^{2+} solutions. Results similar to those of Mason (1980) were also reported by Abe and Ogata (1982) using slices of guinea-pig hypothalamus and by Bourque (1989) using superfused rat hypothalamic explants. More recently, depolarization of MNCs in response to hypertonic stimulation was shown to be retained even when the cells are acutely isolated from the hypothalamus of adult rats (Oliet and Bourque, 1992), providing ultimate evidence that the osmosensitivity of membrane potential is an intrinsic property of these neurons.

Intracellular recordings from MNCs in hypothalamic explants have shown that hypertonic stimuli between +10 and +40 mosmol/kg provoke membrane depolarizations of +2 to +15 mV (Bourque, 1989). Similarly, the average membrane depolarization observed by Mason (1980) for a hypertonic stimulus of +35 mosmol/kg was +13 mV. In the absence of superimposed depolarizing transients, however, depolarizations smaller than 13 mV would not be expected to provoke excitation when elicited from the normal mean resting potential. In apparent agreement with this hypothesis, Mason (1980) has reported that, under conditions where synaptic transmission is blocked, hypertonic stimuli smaller than +35 mosmol/kg fail to evoke firing. The role of the intrinsic depolarizing response to hypertonic stimuli, therefore, is presumably to increase the probability that firing will be triggered by the various depolarizing transients that are superimposed on the membrane potential (Mason, 1980; Leng et al., 1982, 1985; Bourque et al., 1994; Oliet and Bourque, 1994; Bourque and Oliet, 1997).

Osmoreception in MNCs involves a non-selective cation conductance

The first report concerning the possible mechanism underlying the osmosensitivity of rat MNCs came as a result of single electrode voltage clamp experiments using sharp electrode recordings from supraoptic neurons in superfused hypothalamic explants (Bourque, 1989). This study revealed that the depolarization evoked by hypertonic stimulation of MNCs near resting potential is due to the activation of an inward current associated with an increase of membrane conductance. Current–voltage (*I–V*) analysis indicated that the osmotically-modulated current (I_{OSM}) was voltage-insensitive below −50 mV. Extrapolation of the linear portion of the *I–V* curve indicated that the reversal potential (E_{REV}) of I_{OSM} was near −20 mV. Since changes in chloride gradient did not significantly affect E_{REV}, it was proposed that I_{OSM} reflected the activation of a non-selective cationic conductance (G_{CAT}). These results were subsequently confirmed and extended by experiments involving ionic substitution during whole cell recording from MNCs acutely isolated from the supraoptic nucleus of adult rats (Oliet and Bourque, 1993b). Under the conditions of these latter experiments, the E_{REV} of I_{OSM} was near −40 mV. Of particular interest was the discovery that G_{CAT} was not only increased by hypertonic stimulation, as previously reported (Bourque, 1989), but that it could be reduced by hypotonic stimulation (Oliet and Bourque, 1993a,b). Functionally, therefore, the membrane potential of individual MNCs can hyperpolarized by decreases in G_{CAT} during hypotonic conditions or depolarized by increases in G_{CAT} during hypertonicity.

Stretch-inactivated cation channels mediate osmosensitivity in MNCs

Cell-attached single channel recordings from acutely isolated rat MNCs have provided evidence that G_{CAT} is due to the presence of cation-permeable channels in the plasma membrane of these cells (Oliet and Bourque, 1993a, 1996). Under cell-attached recording conditions mimicking those used for whole cell experiments the single channels had a *I–V* slope conductance of 33 pS and carried a cation current reversing near −40 mV (Oliet and Bourque, 1993a; Oliet, 1994). During cell attached patch-clamp recordings, where the perimeter of the

membrane patch was stabilized by the gigaseal formed against the tip of the recording pipette, the probability of opening (P_O) of these channels was decreased by stretching the membrane by applying either negative or positive pressure to the inside of the pipette. The bell-shaped relation between P_O and pipette pressure (Oliet and Bourque, 1993a) thus characterized these channels as being of the stretch-inactivated variety (Morris, 1990). As illustrated in Fig. 2 changes in cell volume evoked by hypertonic or hypotonic stimuli in situ are hypothesized to increase or decrease the lateral tension applied to these channels, thereby resulting in decreases or increases in channel P_O, respectively (for review see Oliet and Bourque, 1994; Bourque and Oliet, 1997).

In agreement with the hypothesized involvement of the 33 pS cation channels in mediating I_{OSM}, changes in channel kinetics provoked by direct mechanical stimulation have been found to mimic those provoked by osmotic stimulation during cell-attached recordings (Oliet and Bourque, 1996). Moreover, the mean open time of these channels is reduced by 50% when exposed to a concentration of gadolinium (Gd^{3+}; 30–40 μM) equivalent to that which inhibits the macroscopic I_{OSM} by one half (40–60 μM) (Oliet and Bourque, 1996).

Relation between P_{OSM} and membrane potential in MNCs

A consequence predicted by the mechanosensitive model of osmoreception is that the membrane potential of MNCs should be regulated over a continuum of values according to the prevailing osmolality. Moreover, since channel activity is inhibited by stretch, I_{OSM} might ultimately approach zero under severe hypotonic conditions. The latter hypothesis was tested directly by monitoring the magnitude of I_{OSM} and G_{CAT} over a wide range of osmolalities. The traces in Fig. 3A show whole cell current responses to 30 mV hyperpolarizing steps applied, under voltage clamp, to an MNC exposed to solutions of differing osmolalities. Increases in the size of the current pulse reflect increases membrane conductance, and therefore G_{CAT}. Note that MNCs display detectable increases in G_{CAT} in response to increases in osmolality as small as +3 mosmol/kg, and that the magnitude of the conductance change is proportional to the amplitude of the osmotic stimulus. The plot shown in Fig. 3B illustrates fractional changes in total membrane conductance measured in response to changes in external osmolality in a group of isolated MNCs. Although the data would probably be best described by a Boltzmann distribution, information concerning maximal conductance attained by hypertonic stimuli is not yet available, making it difficult to define the parameters for such a fit. The sensitivity of the relationship near set-point, however, is adequately approximated by linear regression through the data points obtained at osmolalities ranging between 275 and 325 mosmol/kg (slope = 2.14%/mosmol/kg; $R = 0.96$; $n = 22$) (Oliet and Bourque, 1993b). Interestingly, the decrease in membrane conductance associated with hypotonicity reaches an apparent minimum near 275 mosmol/kg. The high sensitivity (i.e., ±3 mosmol/kg) of G_{CAT} to osmotic stimulation and the similarity of the apparent osmotic thresholds for G_{CAT} (Fig. 3B), MNC firing (Fig. 1B) and VP secretion (Fig. 1A) provide strong support for a functional role of G_{CAT} in the osmotic regulation of MNCs under physiological conditions.

An important factor to consider when evaluating the potential significance of the intrinsic osmosensitivity of MNCs is that the amplitude of the voltage responses to a given I_{OSM} would be inversely proportional to the input conductance of the cell. Moreover, the amplitudes of depolarizing responses to hypertonic stimuli reported in the studies described earlier (Mason, 1980; Bourque, 1989) were measured using intracellular recordings with sharp microelectrodes, where the process of cellular impalement might have artificially lowered the input resistance. Indeed, the input resistance of MNCs appears to be higher when measured by whole cell patch-clamp, which forms a tight seal between the pipette and the plasma membrane (i.e., >1 GΩ), than by intracellular recording (e.g., 905 ± 51 for patch-clamped versus 244 ± 34 MΩ for impaled cells) (Schrader and Tasker, 1997). Fig. 4 shows the changes in membrane potential (V_m) expected to result from changes in P_{OSM} in an idealized MNC. The values of V_m in this figure were calculated using a Goldman–Hodgkin–

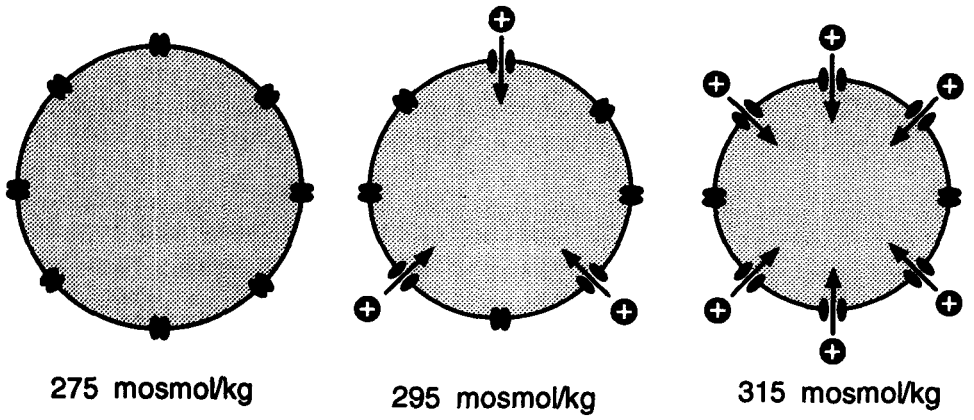

Fig. 2. Stretch-inactivated channels and osmosensitivity in MNCs. The diagram illustrates that cell swelling, resulting from hypotonicity (275 mosmol/kg) causes a decrease in the probability of opening of stretch-inactivated cation channels whereas cell shrinkage resulting from hypertonicity (315 mosmol/kg) provokes an increase in the same parameter.

Katz type approach as described in Appendix A. As is apparent from the graph, changes in G_{CAT} are expected to provoke significant changes in membrane potential during changes in osmolality experienced near the osmotic set-point (about 0.8 mV/mosmol kg^{-1}). The relationship is inaccurate at the extremes, however, because the effects of non-inactivating voltage-gated conductances present

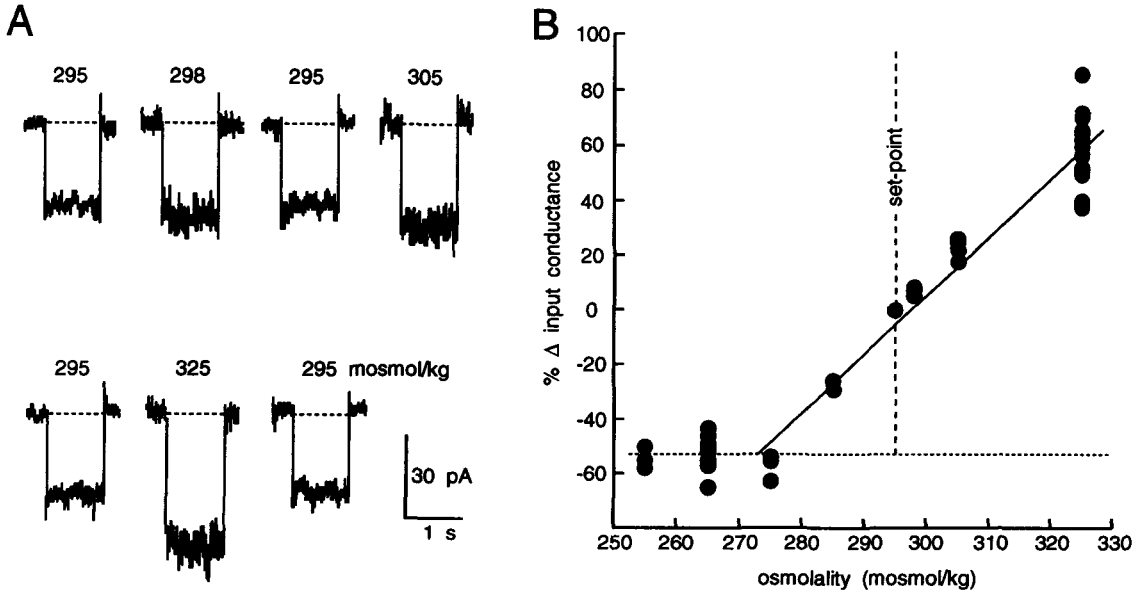

Fig. 3. Osmotic modulation of cation conductance (G_{CAT}) in MNCs. The traces in (A) show current responses to hyperpolarizing voltage commands from −70 to −100 mV applied to a single isolated MNC under whole cell voltage clamp. Each trace shows an example of a response recorded while the cell was bathed in isotonic (295 mosmol/kg) or hypertonic solution (made by the addition of mannitol). The plot in (B) shows the normalized changes (%Δ; relative to the value observed at 295 mosmol/kg) in input conductance provoked by application of various osmotic stimuli to 22 isolated MNCs. The solid line is a linear regression fit of the data points (slope = 2.14 %/mosmol kg^{-1}; $r = 0.96$). Adapted from data published in Oliet and Bourque (1993b).

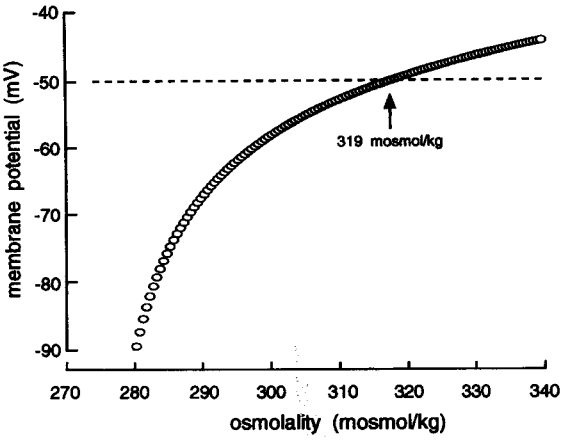

Fig. 4. Hypothetical changes of membrane potential in MNCs as a function of P_{OSM}. The circles show the values of membrane potential calculated from Eq. (1) in Appendix A. The basic features of the relationship are a resting potential of −63 mV at the osmotic set-point (294 mosmol/kg) and the occurrence of voltages above action potential threshold (−50 mV; dashed line) at osmolalities greater than 319 mosmol/kg.

below −80 mV (e.g., Erickson et al., 1993) and above −60 mV (e.g., Stern and Armstrong, 1997) have not been considered. Similar results have been obtained using an iterative model predicting the changes in membrane potential that would be expected to occur as a consequence of varying osmolality in steps of 1 mosmol/kg (Richard, 1997).

Extrinsic factors in the osmotic control of VP neurons

A conclusion drawn from the analysis of the intrinsic osmosensitivity of MNCs is that the ongoing electrical activity observed at P_{OSM} values ranging between 282 and 319 mosmol/kg (Fig. 1B) must be largely driven by depolarizing transients. While a variety of intrinsic depolarizing influences may contribute to the electrical activity of MNCs in this range of osmolalities, spontaneous EPSPs represent the major source of excitatory drive present at subthreshold membrane potentials (e.g., Mason, 1983; Gribkoff and Dudek, 1990; Bourque et al., 1993, 1998; Wuarin and Dudek, 1993; Richard and Bourque, 1995; Inenaga et al., 1997). Since the proportion of EPSPs reaching spike threshold would be expected to steadily increase as a function of P_{OSM}, due to the accompanying depolarization (Fig. 4) (Mason, 1980; Leng et al., 1982, 1985; Bourque et al., 1994; Oliet and Bourque, 1994; Richard, 1997), the intrinsic osmosensitivity of MNCs combined with a constant supply of EPSPs might presumably be sufficient to regulate MNC firing and VP secretion. Recent experiments, however, have revealed that external factors provide more than a simple unregulated source of EPSPs to MNCs. Inputs arising from extrinsic osmoreceptors, in fact, actively contribute to the regulation of VP MNCs both under basal conditions and during osmotic stimulation.

Circumventricular organs as extrinsic osmoreceptors controlling VP release

Evidence that other neurons are involved in the osmotic control of MNCs was first provided by experiments examining the effects of systemic infusions of hypertonic urea on VP release. Because it readily permeates the plasma membrane, cellular shrinkage does not occur in tissues directly exposed to excess urea. This substance, however, poorly permeates the blood-brain barrier (Kleeman et al., 1962; Oldendorf, 1971; Yudilevich and De Rose, 1971) and, when added to the peripheral circulation, progressively extracts water from the cerebral interstitium. Increases in CSF osmolality and sodium concentration ($[Na^+]_o$) resulting from systemic hypertonicity have been found to be similar when evoked by infusion of 1 M NaCl or 2 M urea (e.g., McKinley et al., 1978). Systemic injections of hypertonic urea, therefore, presumably stimulate osmoreceptors lying behind the blood-brain barrier without affecting those that might be present in the periphery. Experiments in humans (Zerbe and Robertson, 1983), dogs (Verney, 1947) and sheep (McKinley et al., 1978) have shown that the use of hypertonic urea to increase P_{OSM} results in a dramatic slowing of the time course and reduction in the amplitude of the VP response compared to that evoked by hypertonic NaCl, despite similar changes in circulating P_{OSM}. Since the centrally-located hypothalamic MNCs are

presumably osmotically stimulated by systemic infusion of hypertonic urea, these results suggest that synaptic inputs originating from osmosensitive neurons located outside the blood–brain barrier are necessary to obtain a prompt and optimal VP response to systemic hypertonicity.

Previous studies have shown that osmoreceptor information can originate in a number of distinct structures in the periphery (for review, see Bourque et al., 1994). The osmoreceptors regulating VP release during systemic osmotic stimulation, however, appear to be located in the brain (Verney, 1947). It has been hypothesized, therefore, that the extrinsic osmoreceptors involved in the regulation of MNCs may be located in one or more of the forebrain circumventricular organs (CVOs; e.g., Thrasher, 1982; Ramsay, 1985); cerebral regions lacking a conventional blood-brain barrier (Weindl, 1973). Although a variety of CVOs (e.g., the subfornical organ; Gutman et al., 1988) may be ultimately found to participate in the osmotic control of VP release (see Bourque et al., 1994), most of the evidence currently available points to the organum vasculosum lamina terminalis (OVLT) as a key structure for osmoreception.

Role of the OVLT as an osmoreceptor regulating VP neurons

OVLT neurons are osmosensitive

Electrophysiological experiments on neurons from the OVLT area in vivo (Honda et al., 1990) and in vitro (Sayer et al., 1984; Vivas et al., 1990; Nissen et al., 1993; Richard, 1997) have shown that a proportion of these cells can respond to changes in osmolality. The osmoresponsiveness of OVLT neurons, which most often consists of excitation during hypertonic stimulation and of inhibition during hypotonicity, is retained during blockade of chemical synaptic transmission in vitro (Sayer et al., 1984; Vivas et al., 1990; Bourque et al., 1994; Richard, 1997), indicating that these neurons are intrinsically osmosensitive. From a functional point of view, OVLT neurons can be excited by hypertonic stimuli as small as +4 mosmol/kg (Gentles, 1997), indicating that the electrical responsiveness of these cells is sufficiently sensitive to contribute to the osmotic regulation of MNCs.

Excitatory and inhibitory connections from the OVLT can regulate MNCs

The existence of OVLT neurons projecting axons to hypothalamic nuclei containing MNCs has been shown anatomically, through the use of anterograde and retrograde tracers (e.g., Camacho and Phillips, 1981; Tribollet et al., 1985; Wilkin et al., 1989; Anderson et al., 1990; Weiss and Hatton, 1990; Oldfield et al., 1994; Armstrong et al., 1996; Voisin et al., 1997) and electrophysiologically, by recording antidromic responses in OVLT neurons following electrical stimulation in the supraoptic nucleus (e.g., Chaudry et al., 1989; Honda et al., 1990; Nissen et al., 1993; Gentles, 1997, Richard, 1997). Moreover, electrical stimulation of the OVLT area in vivo (Honda et al., 1990) and in vitro (Yang et al., 1994; Richard and Bourque, 1995, 1996) has been found to produce both excitatory and inhibitory responses in MNCs of the supraoptic nucleus. The presence of bicuculline-sensitive inhibitory responses following electrical stimulation of the OVLT (Yang et al., 1994; Richard and Bourque, 1995) is consistent with the observation of anterogradely-labelled symmetrical synapses on MNCs following tracer injection into the OVLT (Armstrong et al., 1996). Indeed, previous ultrastructural studies have shown that symmetric synapses formed on MNCs react positively when stained with antibodies against GABA (Theodosis et al., 1986; Buijs et al., 1987; DeCavel and van den Pol, 1990), a transmitter mediating bicuculline-sensitive inhibition in MNCs (e.g., Randle et al., 1986; Randle and Renaud, 1987; Renaud and Bourque, 1991; Wuarin and Dudek, 1993; Brussaard et al., 1997). The presence of excitatory responses to OVLT stimulation, in turn, is consistent with the observation of anterogradely-labelled asymmetric synapses on MNCs following tracer injection into the OVLT (Armstrong et al., 1996). Previous ultrastructural observations have shown that asymmetrical synapses on MNCs contain glutamate-like immunoreactivity (e.g., van den Pol, 1991).

These observations suggest that both excitatory

and inhibitory projections to MNCs may arise from neurons located within the OVLT. In agreement with this hypothesis, experiments in hypothalamic explants have shown that blockade of spike discharge in OVLT neurons, by local infusion of tetrodotoxin (to block voltage-gated Na^+ channels) or GABA (which causes hyperpolarization), causes a significant decrease in the frequency of both spontaneous excitatory postsynaptic potentials (sEPSPs) and inhibitory postsynaptic potentials (sISPSs) recorded from MNCs in the supraoptic nucleus (Richard and Bourque, 1995). Thus, although a proportion of the synaptic inputs between OVLT neurons and hypothalamic MNCs may be relayed polysynaptically (e.g., through the median preoptic nucleus; Honda et al., 1990), direct excitatory and inhibitory projections originating within the OVLT appear to be available for the regulation of MNCs. In apparent agreement with a possible role for these direct projections in the osmotic control of MNCs and VP release, systemic hypertonicity was recently shown to cause enhanced expression of the activity-dependent immediate early gene *fos* in OVLT neurons retrogradely labelled by tracer injections into hypothalamic nuclei containing MNCs (Oldfield et al., 1994).

Electrophysiological experiments in vivo (Honda et al., 1987) and in vitro (Richard and Bourque, 1992) have shown that localized hypertonic stimulation in the area of the ventral AV3V or OVLT excites MNCs. Moreover, lesions of the OVLT (or injections of local anaesthetic into this structure) have been found to suppress or attenuate the excitation of MNCs (Leng et al., 1989; Honda et al., 1990) as well as VP secretion (Thrasher et al., 1982; Sladek and Johnson, 1983; Thrasher and Keil, 1987; Ludwig et al., 1996) that occurs in response to increases in P_{OSM}. A large body of evidence therefore supports the notion that the OVLT may include sensory elements required for transducing changes in systemic osmolality, and for conveying this information to MNCs in the hypothalamic supraoptic and paraventricular nuclei.

Osmotic control of MNCs by the OVLT involves glutamatergic synapses

Although the basis for osmoreception in OVLT neurons remains unknown, recent experiments have clarified some of the mechanisms through which changes in the firing rate of OVLT neurons may regulate MNCs during systemic osmotic perturbation. In particular, while intricate reciprocal connections with other nuclei of the lamina terminalis may play a role in mediating the osmotic control of MNCs (e.g., Chaudry et al., 1989; Honda et al., 1990), experiments using superfused hypothalamic explants have provided information concerning the specific role of the direct OVLT projections to MNCs in the supraoptic nucleus (Richard and Bourque, 1995). As shown in Fig. 5A, this preparation provides an opportunity to perform selective osmotic stimulation of the OVLT area while recording from MNCs in the supraoptic nucleus. Since the intrinsic response of MNCs is not engaged during osmotic stimulation of the OVLT, the membrane potential of these cells remains constant. In these experiments, the membrane potential of MNCs was adjusted by sustained current injection to reveal an initial background firing rate between 0.1 and 5 Hz. Shortly after applying to the OVLT a solution made hypertonic by the addition of mannitol, the firing rate recorded from MNCs was enhanced (Fig. 5B, upper trace). Conversely, applying to the OVLT a solution made hypotonic by removal of mannitol produced a decrease in the rate of firing (Fig. 5B, lower trace). Although the absolute rate of firing was initially determined by the amount of current injected into the cell, changes in the rate of firing provoked by the application of solutions with osmolalities ranging between 275 and 355 mosmol/kg to the OVLT, varied in proportion with the magnitude of the osmotic stimulus (Fig. 5C). Interestingly, firing rate was reduced by application of hypotonic solutions with osmolalities between 280 and 290 mosmol/kg, but was not further lowered by application of hypotonic solutions with osmolalities $\leq$275 mosmol/kg, suggesting that the threshold for the OVLT-mediated mechanism is similar to that of G_{CAT} in MNCs (Fig. 3B).

Close inspection of the membrane potential fluctuations recorded during such experiments have revealed that osmotic stimuli delivered to the OVLT provoke a dose-dependent change in the

frequency of sEPSPs recorded from MNCs (Fig. 6A) but have no effect on the rate of sIPSPs (Fig. 6B). Similar to the measurements of firing rate, osmotic stimuli delivered to the OVLT had little effect on sEPSP frequency at osmolalities ≤275 mosmol/kg. Moreover, osmotically-evoked changes in firing rate correlated well with the associated changes in sEPSP frequency (slope 0.9; $r = 0.7$; $n = 54$), but not with sIPSP frequency ($r = 0.2$). Changes in MNC firing rate evoked by osmotic stimulation of OVLT neurons in superfused hypothalamic explants, therefore, were selectively mediated by changes in synaptic excitation. As shown in Fig. 6C, increases in sEPSP frequency and firing evoked by hypertonic stimulation of the OVLT were reversibly inhibited by bath application of CNQX, an antagonist of the AMPA subtype of glutamatergic ionotropic receptors in MNCs (Hu and Bourque, 1991, 1992). The synaptic drive responsible for the OVLT-mediated osmotic regulation of firing rate in MNCs therefore involves glutamatergic synapses. In agreement with the involvement of glutamatergic synapses in the osmotic control of firing in MNCs, Dyball et al. (1995) have reported that the rate of sEPSP frequency recorded in rat MNCs is increased following hypertonic stimulation in vivo, and that the excitatory response of MNCs to increases in

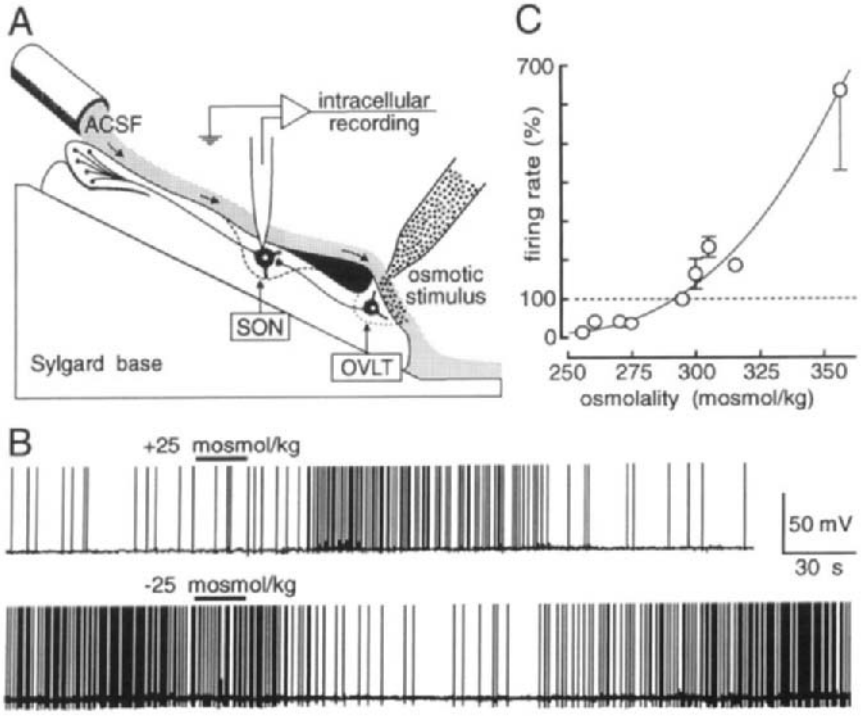

Fig. 5. Effects of osmotic stimulation of the OVLT on the firing rate of MNCs. (A) A diagram of the hypothalamic explant preparation as configured for the present experiments. Note that the delivery of artificial cerebrospinal fluid (ACSF) is arranged so that the hypertonic solution applied to the OVLT does not reach MNCs in the supraoptic nucleus (SON). The traces in (B) are chart recordings of intracellularly recorded membrane voltage in two MNCs. The basal frequency of action potential discharge (vertical deflections) was determined by the amount of sustained current injection adjusted at the beginning of each experiment. Note that application of a hypertonic stimulus to the OVLT (bar, upper trace) causes a reversible increase in firing rate, whereas firing rate is reversibly reduced by application of a hypotonic solution (bar; lower trace). The graph in (C) plots mean (±SEM) normalized changes in firing frequency recorded in a population of MNCs following osmotic stimulation of the OVLT. The values of n for consecutive points between 255 and 355 mosmol/kg were: 7, 1, 5, 1, 58, 4, 28, 7 and 5. Adapted from data published in Richard and Bourque (1995).

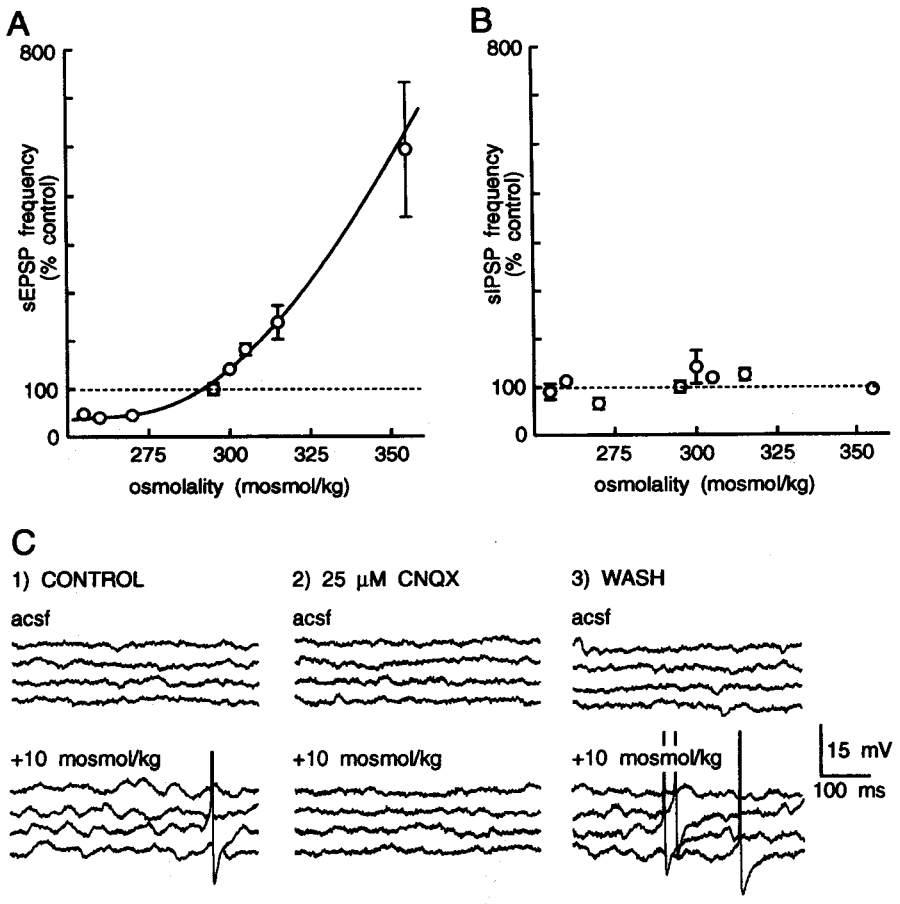

Fig. 6. OVLT-mediated regulation of MNCs via glutamate synapses. The graphs in (A) and (B) plot mean (±SEM) relative changes in spontaneous excitatory postsynaptic potential (A; sEPSP; left graph) and inhibitory postsynaptic potential (B; sIPSP; right graph) frequency occurring in MNCs as a result of osmotic stimulation of the OVLT. All data points express changes in frequency relative to the spontaneous rate observed at 295 mosmol/kg in the same cell. Adapted from data published in Richard and Bourque, 1995). The traces in (C) show high gain excerpts of baseline voltage recorded in a single MNC in the presence of artificial cerebrospinal fluid (acsf, upper sets of four traces) and following stimulation of the OVLT with a hypertonic solution (+10 mosmol/kg; lower sets of four traces). Note that hypertonic stimulation of the OVLT provokes an increase in sEPSP frequency (control, left panels) and that this effect is reversibly abolished by inclusion of 25 µM CNQX (middle panels), an antagonist at AMPA-type glutamate receptors. Vertical deflections are truncated action potentials. Adapted from Richard (1997).

P_{OSM} can be blunted by an intra-cerebroventricular injection of kynuretic acid, an antagonist of glutamate ionotropic receptors.

Conclusions

A variety of experimental approaches have recently provided a wealth of information concerning the neurophysiological basis for the osmotic control of VP-releasing MNCs. In this review we focussed on the recently characterized mechanisms underlying the intrinsic osmosensitivity of MNCs and on the role of the osmosensitive excitatory input derived from OVLT neurons. Exciting results by Hussy et al. (1997) now indicate that intranuclear release of taurine, by glial cells, may contribute

to the osmoregulation of MNCs via effects at glycinergic receptors. While more work is required to complete the identification of cellular mechanisms, interactions, and synaptic pathways contributing to this control, the findings obtained to date indicate that a synergy of intrinsic and synaptic processes may serve to optimize the operation of this osmoregulatory reflex.

The blunting and slowing of the VP response to systemically infused hypertonic urea indicates that osmoreceptors located outside the blood-brain barrier (e.g., in the OVLT) are necessary for the expression of a functional response to hypertonic stimulation. Although the osmotic threshold and sensitivity of G_{CAT} indicates that it is modulated during physiologically-relevant changes in P_{OSM} (Fig. 3), it is not clear if resulting effects on membrane potential (e.g., Fig. 4) play a primary (i.e., conditional) or secondary (i.e., modulatory) role in the osmotic control of MNCs. Specifically, it has not been determined if the absence of this mechanism would abolish, or only compromise, the system's response to an osmotic challenge. Such issues will only be resolved once the effects of peripheral osmotic stimulation have been tested in the absence of coincidental changes in central osmolality or better, when the effects of increased P_{OSM} are examined following an acute knock-out of osmosensitivity in MNCs.

From a biophysical standpoint, the presence of an osmoregulated G_{CAT} under steady-state conditions (Oliet and Bourque, 1993b) elevates the Na^+/K^+ permeability ratio (P_{Na}/P_K) of the resting membrane and therefore provides a steady-state depolarizing influence at the osmotic set-point (e.g., Fig. 4). Thus in the absence of stretch-inactivated cation channels the P_{Na}/P_K value of the resting membrane would be lower and the cell's resting potential would be hyperpolarized compared to controls. This hypothesis is supported by the observation that Gd^{3+}, a blocker of G_{CAT}, provokes membrane hyperpolarization when applied to MNCs bathed in isotonic saline (Oliet and Bourque, 1996). It is possible, therefore, that in the absence of G_{CAT} the membrane potential of MNCs might be sufficiently negative to abolish all spontaneous firing and to render the cells completely insensitive to extrinsic synaptic influences.

Alternatively, changes in membrane potential accompanying variations in P_{OSM} (Fig. 4) may simply influence the proportion of depolarizing transients which successfully provoke excitation (e.g., Leng et al., 1985; Bourque et al., 1994). In this scenario the intrinsic osmosensitivity of MNCs would serve to modulate the gain of the osmoregulatory response (Richard, 1997). The existence and significance of this effect has already received some experimental support. As mentioned earlier, experiments in rats have shown that electrolytic lesions of forebrain regions encompassing the OVLT impair the excitation of MNCs and VP release that normally results from hypertonic stimulation (e.g., Leng et al., 1989; Honda et al., 1990). However the basal firing rate of MNCs under such conditions is severely reduced, with most cells being essentially silent (<0.1 Hz). This finding indicates that a significant fraction of the excitatory input that drives basal firing in MNCs is derived from forebrain regions (see also Richard and Bourque, 1995). When the basal electrical activity of MNCs in OVLT-lesioned rats was rescued by local application of glutamate, the excitatory response of MNCs to systemic increases in P_{OSM} was restored (Leng et al., 1989). This result indicates that the intrinsic responsiveness of MNCs (i.e., the hypertonically-evoked membrane depolarization) may be sufficiently large to modulate the rate of spike discharge in the presence of a basal excitatory drive. Additional studies will be required to quantify the importance of this effect and evaluate its role during synaptic activation.

Appendix A

The values of membrane potential (V_m) as a function of osmolality (π; in mosmol/kg) presented in Fig. 4 were calculated from predicted changes in sodium and potassium conductance (G_{Na} and G_K, respectively) using the following equations:

$$V_m = [RT/F]\log(m/k) \quad (1)$$

where R, T and F have their usual values.

$$m = (G_{Na}[Na^+]_o) + (G_K[K^+]_o) \quad (2)$$

$$k = (G_{Na}[Na^+]_i) + (G_K[K^+]_i) \quad (3)$$

$$[Na^+]_o = (\pi - 15)/2 \quad (4)$$

$$G_{Na} = (G_0 f_1) + (f_2[G_{in} - G_0]) \quad (5)$$

$$G_K = (G_0[1 - f_1]) + ([G_0 - G_{in}][1 - f_2]) \quad (6)$$

$$G_{in} = G_0\,[1 + (k[\pi - 295])] \quad (7)$$

$k = 0.0214$ (slope of the relation between G_{in} and π; Oliet and Bourque, 1993b)

$[Na^+]_i = 10$ mM

$[K^+]_o = 3$ mM

$[K^+]_i = 145$ mM

$G_0 = 1.6$ nS: average input membrane conductance (G_{in}) at rest (i.e., at 295 mosmol/kg) reported during whole cell recordings from MNCs in slices (Wuarin and Dudek, 1993; Li and Ferguson, 1996; Li et al., 1995; Kombian et al., 1997; Li and Hatton, 1997; Schrader and Tasker, 1997).

$f_1 = 0.066$: permeability ratio P_{Na}/P_K of the resting cell membrane, value required to establish a resting potential of −63 mV at osmotic setpoint.

$f_2 = 0.2$: P_{Na}/P_K of the osmotically gated conductance (Oliet, 1994).

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 2.1.2

Neurophysiology of magnocellular neuroendocrine cells: Recent advances

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Magnocellular neuroendocrine cells of the hypothalamic paraventricular and supraoptic nuclei are responsible for most of the vasopressin and oxytocin in the peripheral blood as well as for central release of these peptides in selected brain areas. As the principal component of the hypothalamo-neurohypophysial system, these neurons have been a subject of continual study for half a century. The wealth of solid information from decades of in vivo studies has provided a firm basis for in vitro, brain slice and explant investigations of neural mechanisms involved in the control and regulation of vasopressin and oxytocin neurons. In vitro methods have revealed the presence and permitted the study of monosynaptic projections to supraoptic neurons from the olfactory bulbs, the tuberomammillary nuclei of the posterior hypothalamus and from the organum vasculosum of the lamina terminalis. Such methods have also facilitated the elucidation of the various ionic currents controlling neurosecretory cell activity as well as the roles of calcium binding proteins and release of calcium from internal stores. This review summarizes recent advances in our understanding of the afferent inputs that impinge upon these two cell types, and the cellular and molecular mechanisms intrinsic to these neurons that determine their activity patterns and, in part, their responses to incoming stimuli.

Introduction

Magnocellular neuroendocrine cells (MNCs) of the anterior hypothalamus synthesize and release into the pituitary circulation most, if not all, of the oxytocin (OT) and vasopressin (VP) of neural origin that is found in the peripheral blood. Two prominent condensations of MNCs are the well known and well studied hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei (see Fig. 1). A long established, but frequently overlooked, fact is that these two nuclei together only account for approximately 50% of the OT and VP neurons that project axons to the posterior pituitary (Fisher et al., 1979). The other half of this population is constituted by cells scattered in so-called accessory nuclei and those in small groups lying along blood vessels (Fig. 1). To the extent that we find differences between the similar cell types found in the SON and PVN (in terms of their synaptic inputs, intrinsic properties, etc.), it seems important to remember that the other 50% of the MNCs have gone relatively unstudied, particularly with electrophysiological methods. While it is hoped that what is learned from investigations of the MNCs in the two main cell groups is broadly applicable, there are possible constraints to be considered on any generalizations to the neurons of the entire hypothalamoneurohypophysial system.

MNCs comprise a large and prominent portion of the rat hypothalamic PVN, the remainder being made up of parvocellular elements. By contrast, virtually the entire supraoptic nucleus consists of MNCs. VP and OT neurons tend to occupy different regions in these two nuclei, especially in the PVN, but there is enough mixing of the two cell types that a cell's position within the nucleus is not a certain identifier of its peptide content (Hou-yu et al., 1986). Neither is it possible to discriminate

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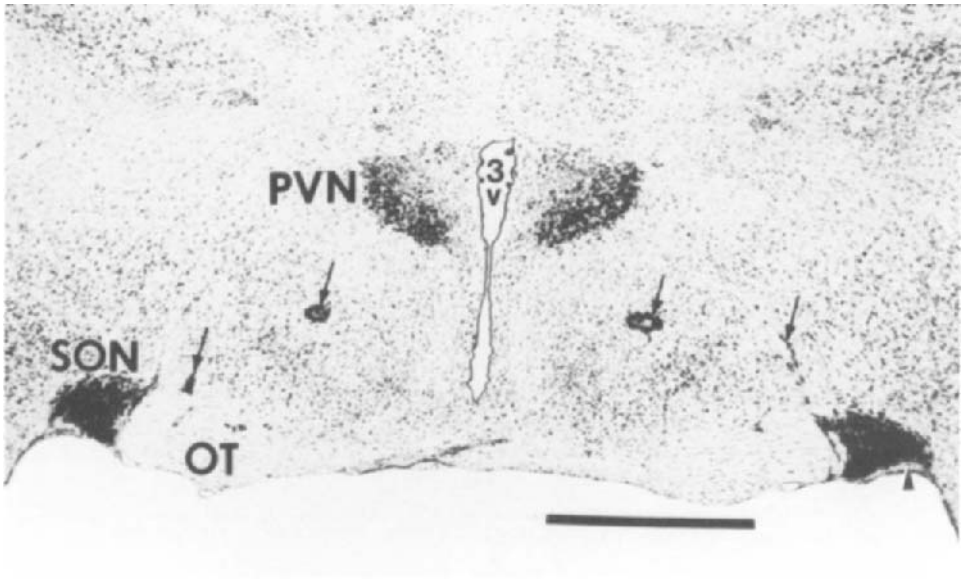


Fig. 1. Coronal section of Nissl-stained rat brain at the level of the paraventricular (PVN) and supraoptic (SON) nuclei in the anterior hypothalamus. Small, internuclear groups of magnocellular neuroendocrine cells (arrows) are also visible. SON dendritic zone is the clear area between the pial surface and the darkly stained cell bodies (arrowhead at lower right). OT, optic tract; 3v, third ventricle. Scale bar = 1 mm.

between OT and VP cells on the basis of simple morphological criteria, such as soma size or shape, dendritic branching, etc. It is often, but not always, possible to distinguish between VP and OT neurons electrophysiologically, by their firing patterns when activated either physiologically or by intracellular injection of depolarizing current. A recent comprehensive review by Armstrong (1995) details the problems and pitfalls involved that can arise in using morphological and/or electrophysiological criteria to discriminate OT from VP neurons.

The highest degrees of certainty concerning the neuron type being studied electrophysiologically comes from those investigations in which recorded neurons are injected with a dye (e.g., Lucifer Yellow) or a tracer (e.g., Neurobiotin) and subsequently identified immunocytochemically for peptide content (e.g., Hatton and Yang, 1989; Armstrong et al., 1994). Even this method does not always result in positive identification of every cell studied, however, as occasionally a neuron with all of the positional, anatomical and physiological characteristics of a VP or OT cell will show no immunoreactivity for either peptide (e.g., Cobbett et al., 1986). This has been interpreted as a failure to detect low levels of peptide expression for a given neuron, rather than representing another cell type. Multiple identifying physiological criteria have been used successfully. These usually involve activating synaptic inputs known to affect selectively either VP or OT cells, such as baroreceptor activation or CCK-mediated inputs, respectively, and using the resulting information in combination with the known firing pattern of the cell type being studied (Harris, 1979; Renaud et al., 1987).

Upon membrane depolarization, VP and OT neurons increase their release (over basal levels) of these peptides from their terminals in the pituitary neural lobe and/or at a variety of central nervous system sites. Under basal physiological conditions, i.e., those in which there is little demand for OT or VP release, MNCs of both types fire slowly and irregularly at rates generally between 0.05 and 0.5 Hz. When activated, OT neurons typi-

cally increase their firing rates, decrease their interspike intervals and display a pattern that is referred to as fast-continuous firing. In this pattern, both the firing rate and the interspike interval distributions are unimodal (Poulain et al., 1988). Activated VP neurons display a contrasting pattern of firing known as phasic bursting, in which the cell alternates between bursts of action potentials and silent periods, each of variable duration, lasting from a few seconds to a minute or more. In this pattern, the firing rates tend to be bimodal. For an individual VP neuron, a burst of action potentials typically follows one or two isolated spikes whose depolarizing afterpotentials (DAPs) summate to initiate a plateau potential upon which the subsequent burst is superimposed (Fig. 2). The rate of firing is typically highest at the beginning of the burst, then slows to a stable rate. The burst terminates prior to the disappearance of the plateau potential. A great deal of effort has been expended over the years in attempts to understand the phenomena of phasic bursting in VP neurons and to determine what mechanisms differentiate OT cells, which generally do not display phasic bursting, from VP cells which do. It is these topics that occupy a large portion of this review. Fortunately, virtually all of these characteristics typical of VP or OT neurons have been observed, quite consistently, both in in vivo and in vitro brain slice preparations (e.g., Brimble and Dyball, 1977; Yang et al., 1995), validating the interpretation of data coming from the more in-depth studies of mechanisms that could only be done in vitro.

Synaptic inputs to MNCs

MNCs of the PVN and SON have long been known to receive synaptic inputs from a variety of sources, including forebrain areas (e.g., medial preoptic region), circumventricular organs (e.g., subfornical organ), and the brain stem (e.g., the nucleus of the solitary tract/dorsal motor vagal nucleus and the A1 region of the ventrolateral medulla). For a review of the evidence for these and other inputs, see Hatton (1990). Recent advances in our knowledge of both the afferents to, and the synaptic physiology of, MNCs has come largely through studies of brain slice and explant preparations. The three sources of synaptic inputs and their physiological effects on MNCs

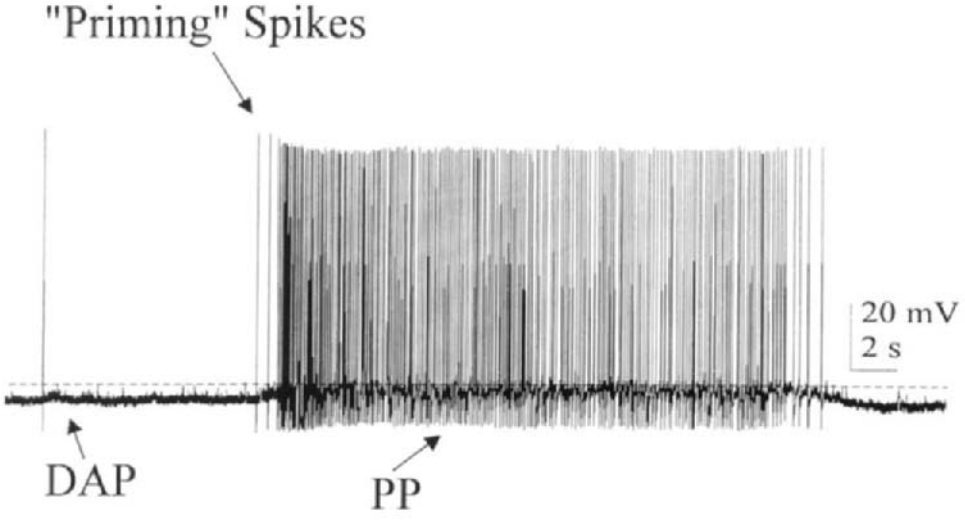


Fig. 2. Single burst of a phasically firing neuron intracellularly recorded in a horizontally cut hypothalamic slice. Individual spikes are followed by depolarizing afterpotentials (DAPs), e.g., the spike occurring ~12 s prior to the burst. Two 'priming' spikes appear to initiate the burst proper as their DAPs summate into a plateau potential (PP; dashed line) upon which all subsequent spikes are superimposed. Firing is more rapid early in the burst than later, with slowing at the end while the membrane is still depolarized. The PP decays as long as 2 s after the final spike.

reviewed briefly here are the main and accessory olfactory bulbs, the tuberomammillary nucleus (TM) and the organum vasculosum of the lamina terminalis (OVLT). For each of these, it is their projections to the SON that have been investigated.

Main and accessory olfactory bulb projections

Antero-grade tracers placed into the main or accessory olfactory bulbs consistently label axon terminals in the SON dendritic zone (see Fig. 1), with only sparse labeling in the region occupied predominantly by cell bodies. In complementary fashion, retrograde tracers injected into the SON are transported back to and fill virtually all mitral cells, as well as some tufted cells, ipsilaterally in both bulbs (Smithson et al., 1989; Smithson et al., 1992). No such projections have been found in the PVN. These findings are most reasonably interpreted as indicating that olfactory output neurons send axon collateral projections to the SON. The parent axons then continue on through the hypothalamus.

Horizontally cut brain slices or explants, prepared such that the piriform cortex containing the lateral olfactory tract (LOT) is spared, permit these pathways from both the accessory and main bulbs to be electrically stimulated while recording synaptically mediated responses from SON neurons. Since the fibers from both bulbs course together in the LOT, this preparation does not allow one to separately stimulate main vs. accessory bulb afferents. Therefore, while it may be that the two bulbs differentially project to subpopulations of SON neurons, our studies to date have not been able to discern this. Electrical stimulation of the LOT has been found to produce fast excitatory postsynaptic potentials (EPSPs) in a high percentage of SON neurons (~85%). On the basis of immunocytochemical identification of dye-injected, recorded neurons, both OT and VP cells appear to be activated by LOT stimulation in roughly equal numbers (Hatton and Yang, 1989). In a small percentage (6%) of neurons recorded, long latency inhibitory postsynaptic potentials (IPSPs) were seen; sometimes these late IPSPs followed the fast excitatory responses. Like the fast excitatory responses, these IPSPs occurred with equal frequency in OT and VP neurons.

The fast EPSPs evoked in SON neurons by LOT stimulation have now been shown to represent monosynaptic inputs mediated by glutamate (Yang et al., 1995). Both continuously firing, putative OT, and phasically firing, putative VP, neurons faithfully followed stimulation frequencies of 50 and 100 Hz, indicating that the connections were monosynaptic. Again for both types of neurons, fast EPSPs could be blocked by the AMPA/kainate receptor antagonists CNQX or NBQX. Slower EPSPs were revealed by bathing the slices in Mg^{2+}-free medium; these could be abolished by the NMDA receptor antagonist, APV. For individual SON neurons, combined application of CNQX and APV was able to completely block EPSPs evoked by LOT stimulation, indicating that OT and VP neurons possess both non-NMDA and NMDA receptors. We concluded that these receptors were most likely located on the dendrites, since the olfactory projections had previously been found to be almost exclusively to the dendritic zone. Although there was earlier, pharmacological evidence for both NMDA and non-NMDA receptors on SON neurons (Hu and Bourque, 1991, 1992), this was the first demonstration that afferent stimulation is capable of activating both subtypes in the same SON neuron regardless of its peptide content. As discussed below, activating these olfactory pathways has been found to have some interesting downstream consequences on direct communication between SON neurons.

Tuberomammillary nucleus projections

This nucleus, consisting of magnocellular neurons located in the posterior hypothalamus, was relatively recently discovered to be the brain's sole source of neuronal histamine (for reviews, see Watanabe and Wada, 1991). TM neurons project to all brain areas so far investigated, making investigations of their actions of general interest. TM axonal projections to the SON have been shown immunocytochemically using antibodies against histamine itself and against its synthetic enzyme, L-histidine decarboxylase (Inagaki et al., 1988; Panula et al., 1989; Decavel and Hatton, 1994).

The SON dendritic zone receives, by far, the heaviest input of histaminergic fibers. Fortunately, horizontally cut brain slices can be prepared so that the projections from the TM to the SON are kept intact, permitting investigations of the effects of synaptically released histamine as well as pharmacological applications. In other brain areas, it has so far been possible only to study the effects of pharmacologically applied histamine. It is also fortunate that a subnucleus of the TM that projects strongly to the SON lies close to the pial surface at the edge of the hypothalamus, and can thus be quite selectively stimulated electrically (Weiss et al., 1989; Yang and Hatton, 1989, 1994; Hatton and Yang, 1996).

Earlier, Dogterom et al. (1976) showed that histamine injected into the SON in rats potently released VP. Using an explant preparation and extracellular recording methods, it was first shown by Armstrong and Sladek (1985) that bath application of histamine produced a long-lasting excitation in SON neurons via H_1-receptor subtype activation. The first physiological evidence that there was a direct, monosynaptic connection between the TM neurons and the VP cells of the SON was obtained via simultaneous, dual intracellular recordings from TM and phasically firing SON neurons (Yang and Hatton, 1989). In this study, depolarizing currents injected into TM neurons evoked, with about 5 ms constant latency, EPSPs or EPSPs leading to action potentials in putative VP neurons of the SON. No responses in TM cells were seen when the SON neuron was depolarized by intracellular current injection. We also confirmed the longlasting excitatory effect of bath-applied histamine on VP cells seen by Armstrong and Sladek (1985). In our study, this was done by showing that action potentials evoked by electrical stimulation of the LOT (as outlined above) or depolarizing current pulses, which normally elicited one or two action potentials, were followed by prolonged after discharges in the presence of histamine at concentrations as low as 10^{-9} M. These responses were blocked by H_1, but not H_2, histamine receptor antagonists. Further understanding of the ionic currents and second messenger signals involved in this histamine-induced, prolonged depolarization has been achieved using patch clamp methods (Li and Hatton, 1996a). This work is discussed below (see Intrinsic mechanisms determining neuronal activity).

An investigation in which the TM was electrically stimulated extracellularly confirmed the excitatory effects seen earlier on VP neurons and revealed that the effect of TM stimulation on OT neurons was inhibition, rather than excitation (Yang and Hatton, 1994). Synaptically released histamine consistently resulted in fast IPSPs in SON neurons that were either immunocytochemically identified as OT cells or displayed fast-continuous firing for at least 3 min, which was the criterion for designating them as putative OT neurons. These fast IPSPs were not blocked by the $GABA_A$ antagonist, bicuculline. They were reduced or abolished by H_2, but not H_1, receptor antagonists and by the chloride channel blocker, picrotoxin, and were reversed in low chloride medium. From these results, we concluded that synaptically released histamine is capable of activating an H_2-receptor mediated chloride conductance in OT neurons of the SON. Histamine has been found to have a similar action on motor neurons of the lobster cardiac ganglion (Hashemzadeh-Gargari and Freschi, 1992). Although there is no direct evidence for GABA release from TM terminals, there is evidence that GABA and its synthetic enzyme, glutamic acid decarboxylase, exist in TM neurons (Senba et al., 1985; Ericson et al., 1991). It is possible, therefore, that the effect observed in our study represents a cooperative action of histamine and GABA, released from the same terminals, on $GABA_C$ receptors, since these are bicuculline insensitive and picrotoxin sensitive (for reviews, see Bormann and Feigenspan, 1995; Johnston, 1996).

Synaptically released histamine thus appears to have opposite effects on OT and VP neurons, inhibiting the former and exciting the latter, suggesting that TM neurons can differentially influence the release of these two peptides. It is not known, however, whether, in addition to H_2 receptors, the OT neurons also have lower affinity H_1 receptors. If so, their activation could produce prolonged depolarization, as seen in VP and other cells, on which inhibitory responses would be superimposed. Alternatively, they may be overriden. This is a topic for future research.

Organum vasculosum of the lamina terminalis projections

This body of heterogeneous cell types and fenestrated capillaries is one of the so-called circumventricular organs of the brain and lies at the anterior base of the third ventricle. Several anatomical studies, employing a variety of retrograde tracers injected into the SON, have produced evidence that at least a subpopulation of OVLT neurons projects to the SON (Wilkin et al., 1989; McKinley et al., 1992; Oldfield et al., 1994). Since anterograde tracer studies have not been reported, the specific region (dendritic or somatic zone) of the SON to which OVLT axons project is not known. It does appear that those OVLT neurons that project to the SON are osmosensitive, as they respond to systemic osmotic stimuli with increased expression of Fos protein (Oldfield et al., 1994). Earlier extracellular recording studies, both in vivo and in vitro, also suggested that many OVLT neurons were responsive to osmotic stimulation (Honda et al., 1990; Vivas et al., 1990). Also, extracellular electrical stimulation of the OVLT region in explants evokes excitatory postsynaptic responses in SON neurons (Yang et al., 1994).

In an elegant study done in explant preparations containing the OVLT and its projections to the SON, Richard and Bourque (1995) selectively applied solutions of varying osmolality to the region of the OVLT and intracellularly recorded responses of SON neurons. Spontaneously occurring EPSPs increased in frequency in a dose-dependent manner with increasing osmolality of the medium perifusing the OVLT. While the medium osmotic pressure and frequency of EPSPs were highly correlated, there was no significant relationship between osmolality and the occurrence of spontaneous IPSPs, suggesting selective mediation of the osmotic responses by increased excitatory drive. Another important result was that virtually all SON neurons were responsive to activation of the OVLT. That is, in the presence of bicuculline to eliminate $GABA_A$ inhibition, 47 of 48 SON neurons responded to electrical stimulation of the OVLT with fast EPSPs. While the SON cells recorded in this study were not immunocytochemically identified, the high proportion of responsive neurons insures that both OT and VP cells were represented. Thus, the OVLT to SON pathway is at least one through which the detection of osmotic changes can be translated into release of both VP and OT.

Possible roles of electrical synapses

The first evidence for electrical interactions between MNCs was published by Andrew et al. (1981), who showed that the fluorescent dye, Lucifer Yellow intracellularly injected into one SON or PVN neuron was capable of being transferred to one or more nearby MNCs to which the injected neuron was coupled. Subsequent studies have provided evidence that the observed dye coupling among MNCs (a) represents electrical coupling (Yang and Hatton, 1988), (b) is exclusively dendrodendritic (Yang and Hatton, 1987; De Zeeuw et al., 1995; Hatton and Yang, 1996), (c) occurs only between neurons containing the same peptide (Cobbett et al., 1985; Hatton et al., 1987), and (d) is mediated by gap junctions formed by connexin32 gap junction proteins (Micevych and Abelson, 1991; Micevych et al., 1996; Miyata and Hatton, unpublished). It has also been shown that the incidence of coupling is (a) positively correlated with physiological conditions that cause increased demand for peptide release, such as dehydration or lactation (e.g., Cobbett and Hatton, 1984; Hatton et al., 1987), (b) influenced by gonadal steroids, as is VP and OT release (Cobbett et al., 1987; Hatton et al., 1992), (c) more extensive when estimated by Neurobiotin, which is thought to cross gap junctions more easily, than by Lucifer Yellow (Hatton and Yang, 1994), and (d) subject to modulation by synaptic inputs (Hatton and Yang, 1990, 1996; Modney et al., 1990).

This last point, the synaptic modulation of coupling, was suggested by the consistent observation that coupling incidence was increased by physiological conditions that create increased synaptic drive. Electrical stimulation of the LOT which, as outlined earlier, carries axonal projections to SON neuronal dendrites, monosynaptically excites virtually all SON neurons via NMDA and non-NMDA receptors. After stimulation of this excitatory amino acid input for 10 min at 10 Hz, there was a 73% increase in coupling incidence in

lactating, but not virgin or male rat SONs. Since basal levels of coupling in male SONs are only slightly below those of lactating rats there appear to be sufficient numbers of junctions in place to be modulated, suggesting that the synaptic action on coupling was ineffective in the males. It is presumed that this effect was due to differential modulation of gap junction channel gating in the lactating rats, perhaps reflecting an upregulation of glutamate receptors, as has been shown to occur in the physiologically activated SON (Meeker et al., 1994; Curras and Decavel, 1995) and which would not have been present in the male or virgin female rats. This could lead to second messenger-mediated effects on the channels, perhaps via increased intracellular calcium ion concentrations ($[Ca^{2+}]_i$).

Stimulation of the histaminergic TM neurons in slices consistently evokes monosyaptically mediated EPSPs in SON vasopressin neurons. Stimulation of this pathway for 10 min at 10 Hz dramatically increased (~200%) coupling among these neurons in SONs from untreated males over those in unstimulated slices (Hatton and Yang, 1996). Both the EPSPs and the coupling increase were blocked by the histamine H_1-receptor antagonist, pyrilamine. Since H_1-receptors are often linked to guanylyl cyclase activation (Greene, 1994), possible second messenger effects were investigated. Addition of 8-bromo-cGMP to the bath increased coupling in the absence of electrical stimulation. Treatment of slices with the guanylyl cyclase inhibitor, LY83583, prevented the stimulation-induced increase in coupling while not interfering with synaptic responses. Finally, the effect of treatment with 8-bromo-cAMP was to suppress coupling below basal levels, suggesting that vasopressin cell coupling may be enhanced or suppressed depending upon the type of receptor activated by a given transmitter/modulator. Such effects have been seen in other systems. In the retina (Hampson et al., 1994) and the striatum (Onn and Grace, 1994), dopamine D_1-receptor activation, linked to adenylyl cyclase activation, uncouples neurons, whereas in the striatum at least, activation of D_2 receptors, which inhibits adenylyl cyclase, increases the incidence of coupling (Onn and Grace, 1994).

Given the short duration of the electrical stimulation that we used, it seems unlikely that our treatments resulted in newly synthesized connexons. Thus, either connexon alignment or some gating of the number of channels open or duration of channel opening probably accounts for the increases in coupling. It is not currently known whether connexon protein phosphorylation state might have been altered by cyclic nucleotide-dependent processes.

While the functional significance of increased coupling between MNCs is not yet well understood, it is likely that the resulting electrical interactions increase the efficiency of peptide release. For OT cells, this could mean that strong coupling facilitates synchronization of firing during milk ejection bursts. For VP cells, which show little synchrony, this could mean that weak coupling desynchronizes the coupled population, producing burst alternation from cell to cell, and the observed relatively constant, non-pulsatile, plasma levels of VP. For more references and further discussion of this topic see Hatton (1997). Clearly, both the fact of coupling and its lability must be considered in any complete explanation of MNC function.

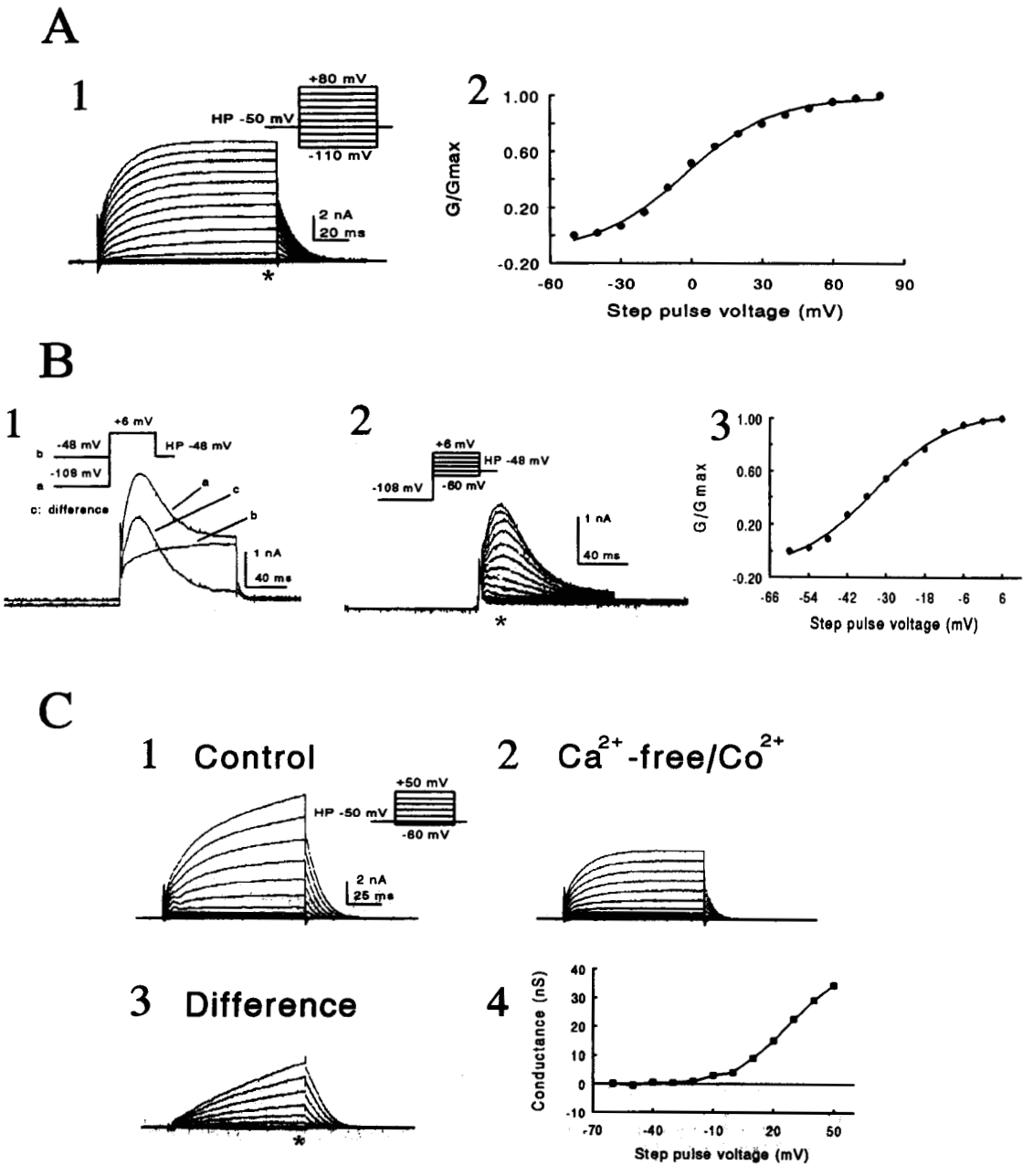
Intrinsic mechanisms determining neuronal activity

Both fiber projections and gap junctions provide MNCs with the means to communicate with neurons within the same nucleus and from other areas. However, responses or consequences of such intercellular interactions depend greatly on the state of neuronal excitability, which is now known to be determined by many cellular elements such as membrane ion channels, cytoplasmic enzyme reactions, intracellular proteins, and Ca^{2+} internal stores. Since the introduction of in vitro methods into studies of MNCs two decades ago, knowledge of these intrinsic mechanisms in MNCs has increased greatly, and hundreds of papers have been generated during this period. Fortunately, there have been several previous reviews of some of this literature (Bourque and Renaud, 1990; Renaud and Bourque, 1991; Legendre and Poulain, 1992; Bourque et al., 1994), so that the present treatment focuses on the latest findings and developments. Here, we describe

properties of channels and cellular elements in an attempt to explain their possible significance in determining MNC excitability.

K^+ currents

K^+ currents play pivotal roles in regulating cellular excitability, as well as firing patterns and frequency, allowing that many transmitters in the CNS effectively influence target cells through modulating the gating of these currents. Like other central and peripheral neurons (Connor and Stevens, 1971a; Meech and Standen, 1975; Thompson, 1977; Galvan and Sedlmeir, 1984), MNCs in



hypothalamic PVN and SON exhibit various kinds of K^+ currents. In primary SON cell cultures, three classic types of currents, i.e., delayed rectifying (I_K), transient (I_A), and Ca^{2+}-dependent ($I_{K(Ca)}$) outward K^+ currents, were first identified according to the differences in kinetics, voltage dependence, Ca^{2+} dependence and pharmacology (Cobbett et al., 1989). Recent extension of these observations to in vitro brain slices obtained from adult rats using whole-cell patch clamp methods have confirmed the presence of these K^+ currents with similar properties in the MNCs of both the PVN (Li and Ferguson, 1996) and SON (Nagatomo et al., 1995). Furthermore, an inward rectifying current (I_h) (Erickson et al., 1990, 1993a), a K^+ leakage current (I_{KL}) (Li and Hatton, 1996a) and a non-inactivating, M-current-like, outward K^+ current (Stern and Armstrong, 1995) have also been reported in MNCs.

The I_K contributes to repolarization of action potentials and reduces neuronal firing rates. It is activated by depolarizing the membrane to more positive than -40 mV and slowly declines during continuous depolarization (Fig. 3A). A rise in $[K^+]_o$, or external application of tetraethylammonium (TEA), a K^+ channel blocker, reversibly suppresses the I_K (Li and Ferguson, 1996), effects which can at least partially explain an increase in spike durations and development of plateau potentials following these treatments in current clamp recording mode (Li and Hatton, 1996b). Although there is a report showing that angiotensin II enhanced the I_K in cultured hypothalamic neurons of neonatal rats (Kang et al., 1993), this effect has not been confirmed in MNCs of adult rats (Nagatomo et al., 1995; Li and Ferguson, 1996).

Following hyperpolarization to remove inactivation, the I_A can be evoked by depolarizing pulses (Bourque, 1988a; Cobbett et al., 1989; Nagatomo et al., 1995; Li and Ferguson, 1996). It begins to be recognizable when clamping the membrane to voltage levels more positive than −65 mV from a holding potential around −90 mV (Fig. 3B). Not only is the I_A rapidly activated, this current can also be inactivated rapidly in a voltage dependent manner. Compared to I_K and $I_{K(Ca)}$, the I_A is more sensitive to 4-aminopyridine (4-AP), another K^+ channel blocker, than to TEA. Observations from different laboratories have not been uniform concerning whether the I_A in MNCs is Ca^{2+}-dependent as well as its responses to Co^{2+} administration. These differences could be the result of various preparations (cell cultures, brain slices or explants; PVN or SON), stage of neuronal development (neonatal or adult animals) and recording methods (sharp or patch electrodes). The I_A participates in the late phase of repolarization of action potentials and delays the occurrence of subsequent spikes (Connor and Stevens, 1971b; Sah and McLachlan, 1992). Because the I_A is substantially inactivated at resting membrane potentials (RMPs) of most MNCs (Bourque, 1988a; Cobbett et al., 1989; Nagatomo et al., 1995; Li and Ferguson, 1996), expression of these functions of I_A in MNCs

Fig. 3. Outward potassium currents from PVN neurons in slices. (A) I_K activation. Whole-cell outward current was evoked by depolarizing voltage steps (A1). The slice was perifused with Ca^{2+}-free medium containing 3 mM EGTA. (A2) Conductance-voltage relation for I_K. The values of membrane currents were measured at the end of voltage steps in A1 (*). Underlying conductances were calculated by dividing current values by driving force, the voltage difference between K^+ equilibrium potential and test pulses. The smooth curve generated by Boltzmann equation. (B) Activation of the I_A. (B1) Whole-cell currents evoked by depolarizing voltage steps with (a) and without (b) a preceding 60 mV hyperpolarization in a PVN neuron. Subtracting (b) from (a) yielded the current difference (I_A) in (c). (B2) Current traces illustrating subtracted I_A activated by various depolarizing voltage steps. (B3) Plots of conductance–voltage relationship for the I_A. Normalized conductances with respect to the maximal conductance (G_{max}) observed in this experiment are plotted against test voltages. Individual conductance (G) was calculated by dividing current values obtained from B2 (*) by driving force. (C) Characteristics of $I_{K(Ca)}$ activation. (C1) Currents evoked by voltage steps during perifusion with medium containing 2 mM Ca^{2+}. (C2) Currents (I_K) evoked by the similar experimental protocol during subsequent perifusion of Ca^{2+}-free medium for the same cell as in C1. Note the difference in current activation, as well as amplitude and duration of the tail currents. (C3) Ca^{2+}-dependent currents obtained by subtracting C2 from C1. (C4) Conductance–voltage curve for $I_{K(Ca)}$. The current values were taken at the end of pulses from the data shown in C3 (*).

depends greatly on hyperpolarization following single or trains of spikes. The hyperpolarization can remove some inactivation and thus make more channels available for subsequent activation by action potentials. Many observations have shown that α-adrenergic agonists (Bourque, 1988b) or angiotensin II (Nagatomo et al., 1995; Li and Ferguson, 1996) reduced the I_A in MNCs, indicating a potential mechanism underlying an increase in excitability following local release of these transmitters (Armstrong et al., 1986; Randle et al., 1986; Okuya et al., 1987; Li and Ferguson, 1993a,b).

The $I_{K(Ca)}$ is activated by depolarizing pulses when Ca^{2+} is present in external medium (Fig. 3C). In comparison with the I_K, this current is characterized by a higher activation threshold, a slower activation time course, little inactivation, and suppression by either depletion of external Ca^{2+} or treatment with Co^{2+} (Cobbett et al., 1989; Li and Ferguson, 1996). The $I_{K(Ca)}$ is involved in repolarizing action potentials and generating afterhyperpolarizations (AHPs) following spike trains, as it has been shown that direct diffusion of Ca^{2+} chelators into MNCs prolongs action potentials and significantly reduces AHPs (Kirkpatrick and Bourque, 1991; Inenaga et al., 1992; Li et al., 1995). Modulation of the $I_{K(Ca)}$ influences spike firing accommodation and phasic or burst firing patterns in MNCs (Andrew and Dudek, 1984; Bourque and Brown, 1987). While clear separation of subtypes of the $I_{K(Ca)}$ has not been attained in MNCs, at least two subtype currents, mediated respectively by high conductance ($I_{BK(Ca)}$ or I_C), and apamin-sensitive small conductance ($I_{SK(Ca)}$), Ca^{2+}-activated K^+ channels, are suggested to underlie those membrane events mentioned. Consistent evidence from several groups (Bourque and Brown, 1987; Fagan and Andrew, 1991; Armstrong et al., 1994) shows that apamin and *d*-tubocurarine effectively block AHPs in rat and cat MNCs. Noradrenaline, 5-HT, ACh, glutamate (Sah, 1996), and neurotensin (Kirkpatrick and Bourque, 1995) can also inhibit the $I_{SK(Ca)}$.

The I_h is an inward current, which operates at RMP levels but is more active when cells are hyperpolarized. It can be enhanced by raising $[K^+]_o$, and eliminated by a specific blocker, Cs^+. Erickson et al. (1993a) reported that neurons displaying the I_h initiated action potentials from more negative levels of the membrane potential than those lacking this current, while other electrophysiological properties, for example, RMPs, input resistances, firing thresholds and firing patterns, are similar between these two groups of MNCs. They proposed that the I_h contributes to rapid restoration of RMPs after prolonged hyperpolarization, such as IPSP bombardment and AHPs. The I_h has been identified in MNCs of guinea-pig (Erickson et al., 1990, 1993a), mouse (Hatton et al., 1996) and rat (Li and Hatton, unpublished data), SON. Upon hyperpolarization, I_h developed in voltage- and time-dependent fashion in guinea-pig and rat MNCs, but showed little time-dependence and was active in more negative voltage ranges in mouse neurons. Interestingly, the I_h can vary greatly in MNCs from different nuclei. Type I neurons in the PVN have been found to exhibit a linear current–voltage (*I–V*) relationship when hyperpolarized, suggesting the absence of I_h (Tasker and Dudek, 1991; Li and Ferguson, 1996).

We have recently demonstrated the presence of the I_{KL} and its modulation by a putative neurotransmitter, histamine, in MNCs of rat SON (Li and Hatton, 1996a). This current was characterized by a linear *I–V* relationship and showed no rectification. Changes in $[K^+]_o$ revealed a reversal potential of I_{KL} close to the equilibrium potential for K^+ (E_K), and treatment with K^+ channel blockers suppressed it. As the I_{KL} is active at RMP levels, it can effectively control cellular excitability. Transmitters and/or modulators like histamine, noradrenaline and ACh have been found to induce prolonged membrane depolarization via inhibiting the I_{KL} in MNCs (Li and Hatton, 1996a) and neurons of the hippocampus (Madison et al., 1987; Benson et al., 1988), dorsal lateral geniculate nucleus (McCormick and Williamson, 1991) and neostriatum (Munakata and Akaike, 1994). The modulating effects of histamine remained even after diffusion of Ca^{2+} chelators into MNCs from recording pipettes to buffer internal free Ca^{2+}, a procedure that usually eliminated Ca^{2+}-dependent membrane events in MNCs, i.e., AHPs and DAPs and phasic firing patterns. Internal diffusion of non-hydrolysable guanosine analogs, GDP-βS or GTP-γS, or

bath treatment with a protein kinase inhibitor, H_7, blocked histamine's effects on I_{KL} (Li and Hatton, 1996a). Prior activation of protein kinase C also occluded further suppression of I_{KL} by histamine. These results suggest that a Ca^{2+}-independent, G-protein mediated activation of protein kinase C is possibly responsible for inhibitory modulation of I_{KL} in MNCs by histamine.

Stern and Armstrong (1995) reported a time-dependent voltage rectification induced by injecting hyperpolarizing pulses into most OT neurons. These cells, obtained from virgin female or lactating rats, were current-clamped above −50 mV, and perifused with a medium containing TTX. The rectification was present above −75 mV and decreased in amplitude as hyperpolarizing pulses approached the E_K. It was larger when MNCs were clamped either to more positive potential levels or for a longer time. It was blocked by TEA and low doses of Ba^{2+}, but not by the specific blocker for I_h, Cs^+. Based on these findings, therefore, the rectification has been proposed to be the result of deactivation of a voltage-dependent, non-inactivating outward current. In fact, it resembles the M-current identified in central and peripheral neurons (Brown and Adams, 1980). Since no VP cells display this rectification, it is possible to use such membrane property as a fingerprint for most OT neurons in female rats (Stern and Armstrong, 1996). Functional roles for this rectification, as well as its modulation by transmitters and modulators, remain to be clarified.

Ca^{2+} current (I_{Ca})

Although an increasing body of evidence has suggested importance of Ca^{2+} influx in controlling cellular excitability and firing patterns, much less about the I_{Ca} and related physiology in MNCs is known so far in comparison with K^+ currents. According to inactivation kinetics, voltage dependence and pharmacology, L-, N-, P-, Q- and T-type Ca^{2+} channel currents have been classified in acutely-dissociated MNCs from rat SON, as in other neuronal preparations. Since there was a significant amount of I_{Ca} unblocked following combined treatments of currently available channel blockers (Fisher and Bourque, 1995a; Foehring and Armstrong, 1996), it is predictable that more types of Ca^{2+} channel currents will be isolated with the development of new specific blockers.

L-type channel currents expressed in the somata of MNCs had small, slowly inactivating and large, non-inactivating components (Fisher and Bourque, 1995a). The latter component was evoked by depolarization above −50 mV and reached a maximal value around −20 mV. Nifedipine, a 1,4-dihydropyridine, rapidly blocked this component. Less than 30% of whole-cell I_{Ca} activated by clamping the membrane to −10 mV from a holding potential of −80 mV or −90 mV is due to L-type channel currents (Fisher and Bourque, 1995a; Foehring and Armstrong, 1996). Even so, L-type currents with such low thresholds near the RMP, can effectively participate in regulating cellular excitability. We have recently shown that nifedipine, as well as N-type Ca^{2+} channel blockers, suppressed the DAPs and phasic firing patterns in MNCs (Fig. 4), supporting involvement of this type current in mediating Ca^{2+}-dependent membrane events. Furthermore, L-type channel current is possibly subjected to modulation by transmitters that stimulate MNCs.

N-type channel current in MNCs displayed a higher activation threshold (above −40 mV) than L-type channel current, and was sensitive to ω-conotoxin GVIA (CgTx), a specific N-type Ca^{2+} channel blocker (Fisher and Bourque, 1995a; Foehring and Armstrong, 1996). Fisher and Bourque (1995a) reported blockade of sustained (by ~40%) and slowly inactivating (by ~60%) components of whole-cell I_{Ca} following CgTx treatment. Considering the potent inhibition of DAPs by CgTx (Li and Hatton, 1997), these findings imply N-type channel mediation of Ca^{2+} influx during action potentials.

T-type current has been shown in MNCs of the guinea pig (Erickson et al., 1993b) and rat (Fisher and Bourque, 1995b). This current was evoked by depolarization above −65 mV and then rapidly inactivated. It was inhibited by Ni^{2+}, a specific blocker of T-type Ca^{2+} channel, in a dose-dependent manner. Although closely associated with DAPs and phasic firing in guinea pig MNCs (Erickson et al., 1993b), T-type currents, and their products observed in current clamp recording, i.e.,

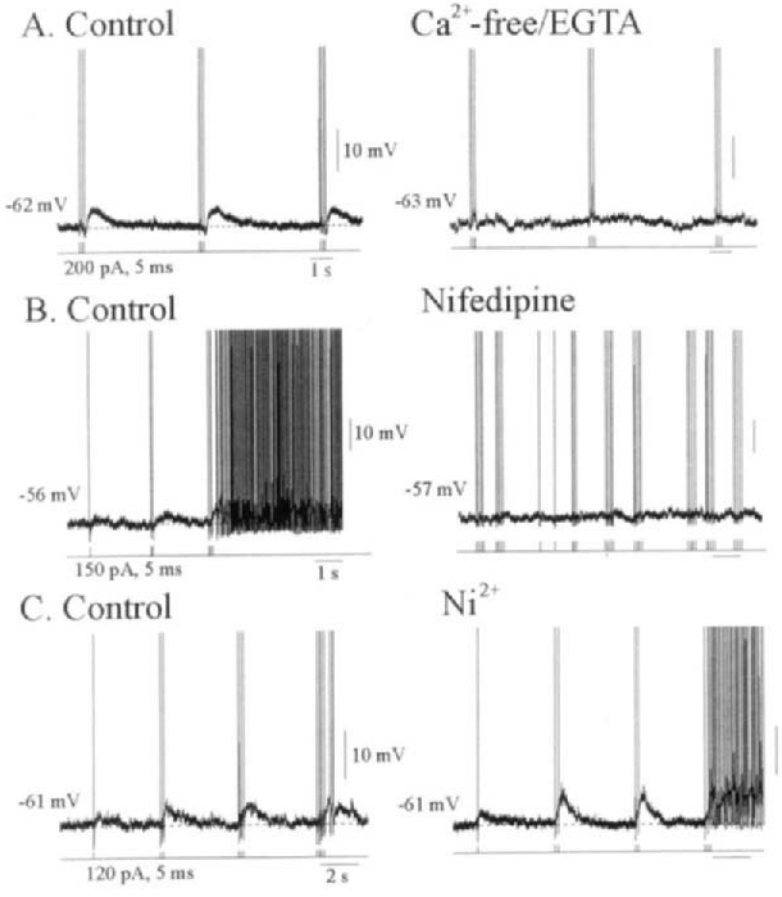


Fig. 4. Requirement of Ca^{2+} influx for DAP formation. (A) Voltage record demonstrating three spike-evoked DAPs of constant size during perifusion with control medium containing 2.4 mM Ca^{2+} (left). These DAPs, however, were eliminated 10 min after replacement of Ca^{2+} in medium with equal concentration of EGTA (right). (B) Records from another cell showing inhibitory effects of an L-type Ca^{2+} channel blocker on DAPs. In control (left), DAPs were repeatedly evoked, and their amplitudes and durations increased with the number of preceding spikes. Bath application of 10 μM nifedipine eliminated DAPs within 10 min (right). Note that no DAP was induced even though more spikes were applied. (C) Records acquired from an SON cell demonstrating that perifusion of medium containing 0.2 mM Ni^{2+}, a T-type Ca^{2+} channel blocker, for 10 min failed to suppress, but actually enhanced, DAP amplitudes (C, right). Note that in this and following figures, the effects of treatments were evaluated by comparing amplitudes and durations of DAPs evoked by the same number of spikes.

low-threshold spikes, are usually absent or small in rat MNCs (Tasker and Dudek, 1991; Oliet and Bourque, 1992; Li and Ferguson, 1996). Furthermore, DAPs and phasic firing remained after treatment of rat MNCs with Ni^{2+}, indicating ionic mechanisms other than T-type current responsible for these events (Fig. 4). As voltage pulses activating T-type current can also evoke the I_A in MNCs, an interactive relationship between these two currents has been revealed (Fisher and Bourque, 1996).

Using a low dose (50 nM) of ω-Agatoxin IVA (from the venom of a funnel-web spider), P-type channel current was isolated in MNC soma (Fisher and Bourque, 1995a; Foehring and Armstrong, 1996). This non-inactivating current in the soma

had a high activation threshold (−30 mV) and accounted for ~20% of total I_{Ca}. Application of ω-conotoxin MVIIC, a non-specific Q-type channel blocker, revealed a possible existence of Q-type current (Foehring and Armstrong, 1996). In axon terminals of MNCs, a high threshold, inactivating I_{Ca} was also identified, which differed from the P-type and the Q-type currents (Fisher and Bourque, 1995b). Further study, of course, is needed to understand the functional significance of these subtypes of I_{Ca} in MNCs.

Na^+ current (I_{Na})

Two classes of TTX-sensitive inward I_{Na} are present in MNCs, i.e., fast (I_{NaF}) and persistent (I_{NaP}) currents. The I_{NaF}, well-known to be responsible for fast action potentials, is characterized by rapid activation and inactivation. For example, it reached a peak within 1 ms of the onset of voltage steps and then declined to a steady-state level within 5 ms in cultured MNCs from the neonatal rat (Cobbett and Mason, 1987). Recently, a low threshold, non-inactivating inward I_{Na} has been identified in MNCs of adult SON (Li and Hatton, 1996b). It was elicited by depolarization from −85 mV to −55 mV or more positive levels and had no inactivation during voltage pulses of several hundreds of milliseconds (Fig. 5). Voltage ramp tests revealed an activation threshold around −56 mV. This current is very similar to the I_{NaP} found in neurons of guinea pig thalamus and cat sensorimotor cortex (Jahnsen and Llinas, 1984; Stafstrom et al., 1985). The I_{NaP} has been proposed to depolarize the membrane to the threshold for I_{NaF} activation and, therefore, plays a decisive role in regulating cellular excitability. The gating of Na^+ channels can be affected by divalent ions inside and outside cells (see Moczydlowski and Schild, 1994 for review). We found that depletion of external Ca^{2+} induced a shift of I_{NaP} threshold toward more negative potentials by >10 mV and enhanced the I_{NaP}, allowing rat SON cells to display oscillatory bursting activity (Li and Hatton, 1996b). Addition of Co^{2+}, Ni^{2+} or Mg^{2+} into Ca^{2+}free-medium, or chelation of intracellular Ca^{2+} with BAPTA, all eliminated the oscillation.

Non-selective cationic current (I_{NC})

Investigations of osmosensitivity as well as modulating effects of putative transmitters have led to the discovery of the I_{NC} in MNCs (Yang et al., 1991, 1992; Bourque et al., 1994). The current showed a linear *I–V* relationship over the range from −120 mV to −40 mV, suggesting independence of the membrane potential. Extrapolation revealed its reversal potentials to lie between −45 mV and −15 mV. Replacement of external Na^+ always induced a hyperpolarizing shift of reversal potentials, while an increase in $[K^+]_o$ shifted the reversal potentials toward more positive values. External application of TTX, a specific Na^+ channel blocker, did not abolish I_{NC}. These findings indicate that the channel associated with I_{NC} has little or no selectivity over monovalent cations and can pass at least two different ions, i.e., Na^+ and K^+.

The I_{NC} in MNCs has attracted much attention after in vitro studies demonstrated that putative transmitters and/or modulators, such as angiotensin II, dopamine, ATP, UTP, CCK and neurotensin, induced prolonged depolarization in MNCs via activation of this current (Yang et al., 1991, 1992; Hiruma and Bourque, 1995; Kirkpatrick and Bourque, 1995; Chakfe and Bourque, 1996). These findings are important for understanding the mechanisms underlying slow and long-lasting responses of MNCs to many central substances. Due to various membrane receptors involved, an arduous task for the future is to investigate the intermediate processes between binding of these receptors with ligands and I_{NC} activation. While external Ca^{2+} was not essential in I_{NC} activation following receptor challenges, adequate $[Ca^{2+}]_i$ was critical, as it was shown that dopamine-induced depolarization remained in Ca^{2+}-free medium, but was cancelled after BAPTA injection into MNCs (Yang et al., 1991). This suggests that either the I_{NC} is Ca^{2+}-dependent and/or dopamine induces Ca^{2+} release from internal stores, which then modulate the I_{NC}. Whether a common pathway exists, involving direct actions of Ca^{2+} or protein kinase activation/channel phosphorylation in I_{NC} modulation by these putative transmitters, should be considered.

Another exciting discovery regarding the I_{NC}

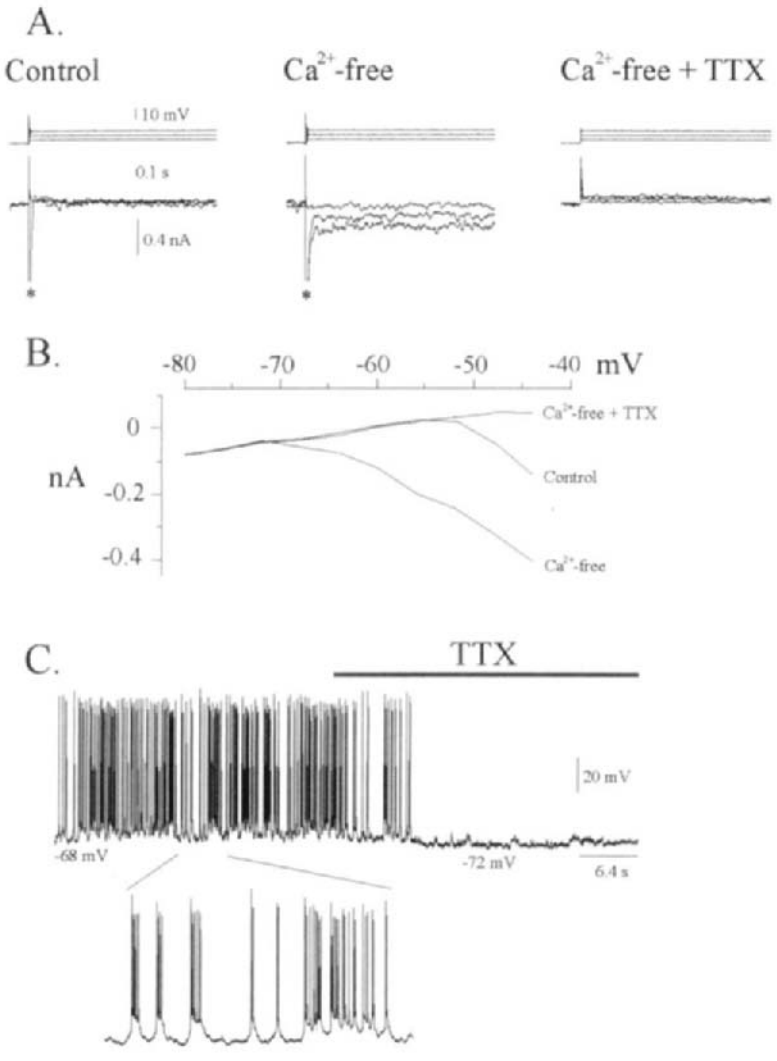


Fig. 5. Enhancement of the persistent Na^+ current (I_{NaP}) following removal of external Ca^{2+}. (A) Current traces evoked by depolarizing commands clamping the membrane potential from -70 mV (holding potential) to −64, −56 and −48 mV. Perifusion of slices with Ca^{2+}-free medium for 8 min induced an inward current, with little inactivation (middle), which was quite small and required larger voltage steps to activate in control (Ca^{2+}-containing) medium (left). TTX (1 μM), added to Ca^{2+}-free medium, blocked the I_{NaP} within 3 min (right). Asterisks indicate the fast Na^+ current characterized by rapid inactivation. Throughout this experiment, TEA (20 mM) and 4-AP (10 mM) were added to perifusion media, and AHP elimination and prolonged spike repolarization were observed before perifusion of Ca^{2+}-free medium. (B) *I–V* curve demonstrating that depletion of external Ca^{2+} enhances the I_{NaP} and shifts its activation threshold toward the hyperpolarizing direction. Measurements were made at the end of 500-ms duration voltage steps. (C) Dependence of bursting on Na^+ influx. TTX (2 μM) application (bar) abolished Ca^{2+} depletion-induced bursting and caused membrane hyperpolarization. Lower trace is segment shown at faster sweep speed.

came from research on osmosensitivity in MNCs. Those interested in this topic are encouraged to read a comprehensive review by Bourque et al. (1994). In brief, osmotic stimulation was found to influence

membrane potentials of MNCs via modulating the I_{NC}; hyperosmotic saline activated the I_{NC} to depolarize the neurons, while hypoosmotic saline inhibited this current and induced membrane hyperpolarization. Changes in I_{NC} conductances during osmotic stimulation were made possible by stretch-inactivating cationic channels (SICs), now identified in the membrane of MNCs (Oliet and Bourque, 1993). More SICs are thought to be in an open state when hyperosmotic stimuli induce MNC shrinkage. This would allow more cations to enter the neurons and produce an inward current and depolarization. In contrast, cell swelling during hypoosmotic stimuli shuts off (or inactivates) SICs so that fewer cations than in the normal (control) condition can enter the cells and cause an apparent outward current and hyperpolarization.

It is quite possible that the SICs are not the channels responsible for the I_{NC} activated by transmitters mentioned above. If more than one type of channel is involved in generating the I_{NC}, then studies of biophysical and molecular differences between them should be revealing.

Ca^{2+} Binding proteins

In addition to the induction of membrane depolarization, a rise in $[Ca^{2+}]_i$ following Ca^{2+} influx through voltage gating or receptor-coupled channels can modulate other ionic channels, trigger Ca^{2+} release from internal stores, increase hormone and transmitter release, and promote biochemical processes including gene transcription and protein synthesis. In MNCs, an adequate elevation in $[Ca^{2+}]_i$ is required to generate Ca^{2+}-dependent DAPs and phasic firing patterns (Andrew and Dudek, 1983; Inenaga et al., 1992; Li et al., 1995). The peak and time course of elevated $[Ca^{2+}]_i$ are known to be controlled by two major cellular elements, i.e., Ca^{2+} pumps, which expel free Ca^{2+} ions into interstitial space and refill internal stores, and cytoplasmic Ca^{2+} chelators, Ca^{2+} binding proteins. Since there are scarce data regarding Ca^{2+} pumps in MNCs, we discuss only the role of Ca^{2+} binding protein here.

Calbindin-D_{28k} (calbindin) is a member of the Ca^{2+} binding protein family, which has been recently found in the CNS including MNCs of the PVN and SON, and proposed to function as an endogenous buffer (Jande et al., 1981; Celio, 1990). With six high affinity Ca^{2+} binding sites, calbindin attenuated Ca^{2+} transients due to Ca^{2+} entry through membrane channels (Chard et al., 1993; Lledo et al., 1992). In certain pathological situations, for example, epilepsy, aging and Alzheimer's disease, a loss of calbindin was discovered in brains, suggesting a protective effect of the protein against neurotoxicity (Iacopino and Christakos, 1990; Kohr et al., 1991). Since many investigations revealed a variable amount of calbindin in MNCs of the SON (Sanchez et al., 1992; Arai et al., 1993), we hypothesized that calbindin can determine the firing activity in these neurons (Li et al., 1995). That is, that MNCs containing high concentrations of calbindin do not display Ca^{2+}-dependent DAPs and phasic firing patterns, while DAPs and phasic firing are present only in MNCs with low levels of calbindin expression.

When diffused from recording pipettes into MNCs, purified calbindin reduced amplitudes of DAPs by ~90% and eliminated phasic firing within 10 min (Fig. 6A). Similar effects were observed following internal application of bovine Ca^{2+} binding proteins with analogous buffering capability or of a well-defined Ca^{2+} chelator, BAPTA. Diffusion of a specific antibody against calbindin revealed DAPs and phasic firing patterns in MNCs that previously did not display DAPs and fired continuously (Fig. 6B). When normal serum, an antibody against endogenous neurophysin or an antibody against glial fibrillary acidic protein, was diffused into MNCs, continuous firing patterns remained and no change in DAP amplitudes was seen, suggesting that the effects of anti-calbindin are specific and result from direct interference of Ca^{2+} binding capability of calbindin. When the anti-calbindin was used to stain the SON, we also found more strongly immunoreactive MNCs located in the dorsal area, where most of cells are known to contain OT. These results fully support our hypotheses that calbindin has a critical, physiological function to control neuronal firing activity in MNCs. Phasically firing MNCs with DAPs lack or have low concentrations of calbindin, and thus higher $[Ca^{2+}]_i$ can be reached after Ca^{2+} influx. MNCs containing more cytoplasmic calbindin

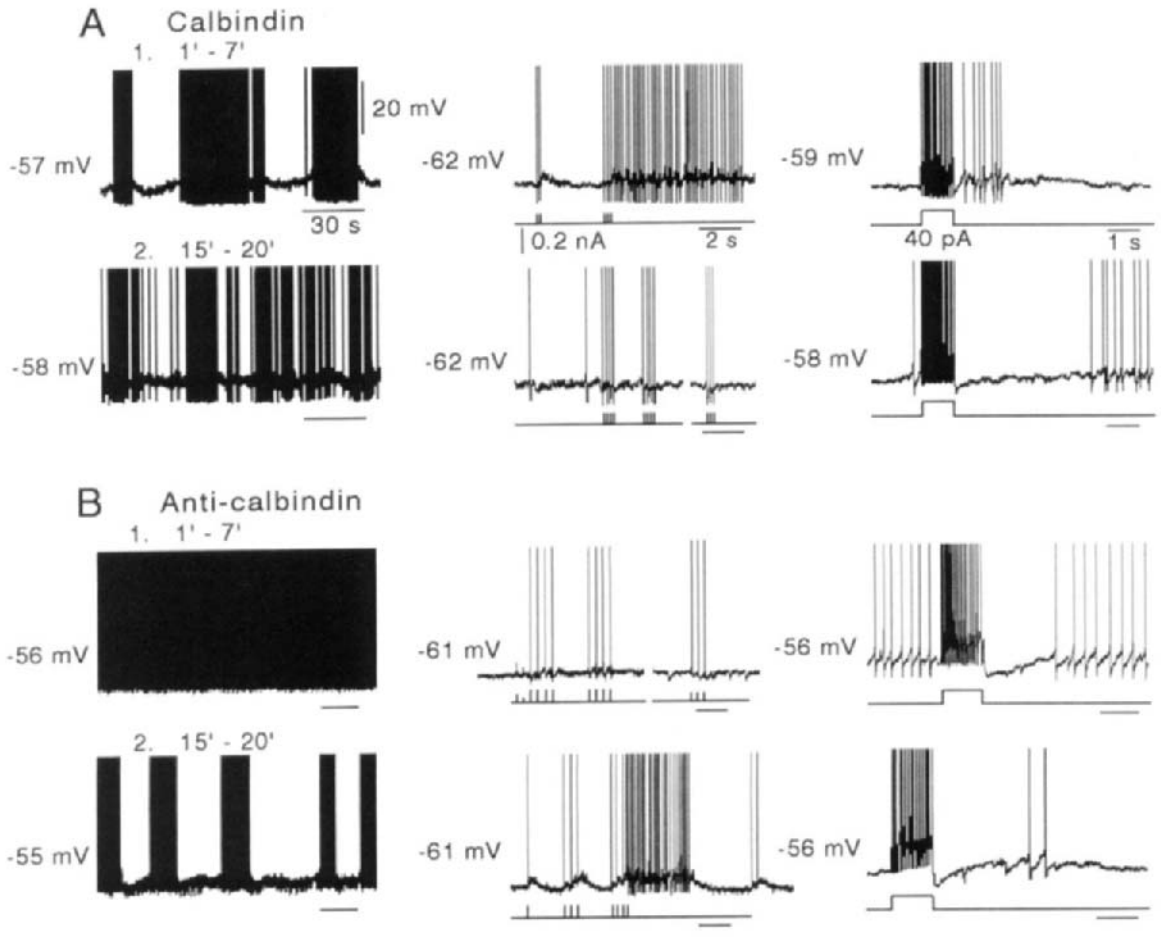


Fig. 6. Endogenous calbindin suppresses DAPs and phasic firing. Left traces: Spontaneous activity; center and right traces: evoked activity. Times shown above voltage traces are periods when data (left, middle and right) were acquired. Calbindin (A) or anti-calbindin (B) was diffused into MNCs from recording pipettes. (A1) Phasic firing and DAPs were observed immediately following membrane rupture. (A2) After 15 min, continuous activity replaced phasic firing, and DAPs were eliminated. The time between two voltage traces in A2 (middle) is 12 s. (B1) Recordings showing successive discharge of spikes and lack of DAPs early after membrane rupture. (B2) By 15 min of recording, phasic firing and DAPs were discovered in this same neuron. In B1 (middle), the time between two voltage traces is 10 s.

only fire action potentials continuously because DAPs and phasic firing cannot be generated due to controlled $[Ca^{2+}]_i$.

Although little information about other members of Ca^{2+} binding protein family in MNCs is available now, functional roles in modulating Ca^{2+}-dependent events similar to that of calbindin can be expected. It was found that intracellular diffusion of an antibody against calretinin occasionally revealed DAPs and phasic firing in MNCs recorded from rostrodorsal regions of the SON (Li et al., 1995). Whether Ca^{2+} binding proteins influence other responses induced by raised $[Ca^{2+}]_i$ is a fascinating subject for future study.

Internal Ca^{2+} stores

As mentioned above, Ca^{2+} influx can evoke Ca^{2+} release from internal stores, a process so-called Ca^{2+}-induced Ca^{2+} release (CICR), and prolong

increases in $[Ca^{2+}]_i$. In heart and smooth muscle cells, the CICR has been known for years to be essential in excitation-contraction coupling. Recent investigations disclose that the CICR is present in CNS neurons and participates in initiating Ca^{2+}-dependent membrane responses (Henzi and MacDermott, 1992), including slow AHPs (Sah and McLachlan, 1991; Yoshizaki et al., 1995).

Our study of possible involvement of the CICR in the generation of DAPs was prompted by finding a disparity of time courses between DAPs and Ca^{2+} transients evoked by action potentials. In general, DAPs following single spikes reach a peak value several hundreds of milliseconds after spike repolarization, and last one or two seconds. Time to peak $[Ca^{2+}]_i$ due to Ca^{2+} entry during action potentials, however, is much shorter; it usually reaches its maximum at the ends of spikes and then declines swiftly. As shown in dentate granule neurons of the hippocampus, where DAPs are mainly dependent on Ca^{2+} influx through T-type Ca^{2+} channels (Zhang et al., 1993), and in molluscan neurons, where fast AHPs are induced by activation of $I_{K(Ca)}$ (Barish and Thompson, 1983), time courses of Ca^{2+}-dependent membrane events faithfully reflect the changes in $[Ca^{2+}]_i$ during action potentials. These DAPs or AHPs were shown to display a peak immediately at the end of action potentials and then decay rapidly within 100 ms. We hypothesized, therefore, that the CICR is another source of Ca^{2+} which maintains, or slows the decline in, Ca^{2+} transients following spikes, and thus allows long durations of DAPs. With the availability of agents modulating Ca^{2+} release from internal stores, we undertook experiments to test this hypothesis (Li and Hatton, 1997).

In MNCs, bath perifusion of ryanodine or dantrolene, blockers of Ca^{2+} release channels, was found to significantly reduce DAP amplitudes and to shorten their durations by ~50%. These effects were mimicked by direct diffusion of ruthenium red, a dye with similar blocking effects on Ca^{2+} release channels, into MNCs with DAPs (Fig. 7A), confirming intracellular actions of ryanodine and dantrolene. Depletion of internal Ca^{2+} stores with either thapsigargin or cyclopiazonic acid, agents that directly inhibit Ca^{2+}-ATPase pumps in the cytoplasmic membranes and thus reduce Ca^{2+} uptake into internal stores, also suppressed DAPs by approximately 50%. Enhancement of Ca^{2+} release by bath application of caffeine induced an increase in DAP amplitudes and durations (Fig. 7B). Importantly, blockade of Ca^{2+} release was also accompanied by the elimination of phasic firing patterns. These results suggest the presence of internal stores in MNCs and functional roles in the generation of DAPs and phasic firing. The CICR may explain, at least partially if not completely, the long durations of DAPs following brief Ca^{2+} influx evoked by single action potentials.

It is possible that, besides the CICR and calbindin, other factors or mechanisms can also take part in the formation of DAPs via regulating $[Ca^{2+}]_i$. For example, the number and efficacy of Ca^{2+} pumps might greatly influence $[Ca^{2+}]_i$ and DAP durations. Once bound to membrane receptors, transmitters or modulators often induce production of inositol-1,4,5-trisphosphate (IP_3) and increase basal $[Ca^{2+}]_i$ through promotion of Ca^{2+} release from IP_3-sensitive stores; these effects can facilitate DAP generation for a prolonged time.

Concluding remarks

Many advances have recently been made in our knowledge of the intrinsic mechanisms underlying the excitability and activity patterns of VP and OT neurons of the hypothalamic PVN and SON. Some of the evidence for these advances has been reviewed here. These include demonstrations of roles for K^+ currents, I_{NaP} or nonselective cation currents in prolonged excitability as well as in governing firing rates, the influences of CICR in sustaining DAPs, and the effects of extracellular Ca^{2+} concentration and gap junctional communication on the stability of membrane potentials in VP neurons. Much work remains to be done in order to gain a full understanding of how these intrinsic mechanisms interact to produce the activity observed in this system under various physiological conditions. Other work has advanced our appreciation for recently discovered synaptic inputs (e.g., from the OVLT) and the actions of newly disclosed neurotransmitters and modulators that may have similar or opposite actions on VP vs. OT neurons (e.g., synaptically released histamine). Further,

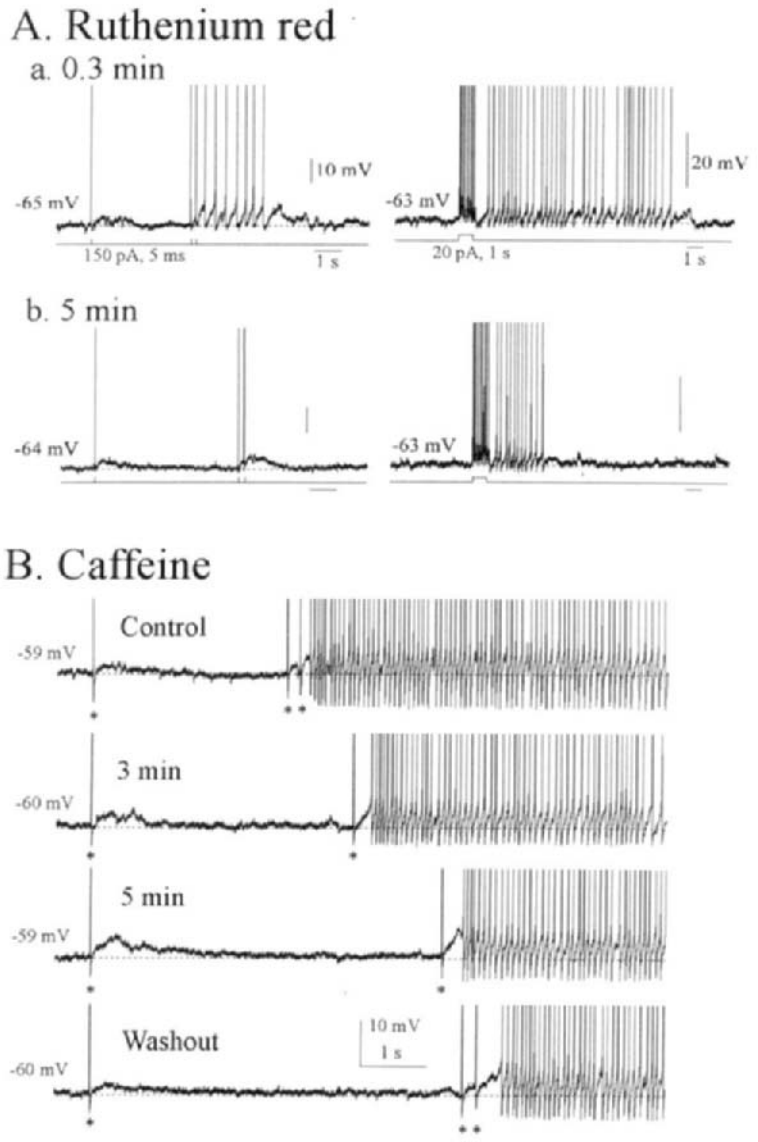


Fig. 7. The effects of manipulating Ca^{2+}-induced Ca^{2+} release (CICR) on DAP generation. (A) Suppression of DAPs by intracellular diffusion of ruthenium red, a blocker of ryanodine receptors. Voltage records obtained from a cell in control medium using an electrode containing 20 μM ruthenium red. DAPs were observed 0.3 min following membrane rupture (Aa), but greatly reduced 5 min later (Ab). Again, 1-s duration pulses were applied to show profound change in DAP summation after the blockade of CICR. (B) Enhancement of DAPs by caffeine. Whole-cell records obtained from a cell showing DAPs before caffeine perifusion (B, top). Asterisks indicate spikes evoked by 140 pA, 5-ms duration current injections. After treatment with 10 mM caffeine for 3 to 5 min, DAPs were prominently enlarged, and often DAPs following single spikes were big enough to trigger bursts of spikes (B, middle rows), whereas in control (B, top), two spikes were required. These influences of caffeine on DAPs largely disappeared 20 min after washout (B, bottom).

demonstrations that at least excitatory synaptic inputs can dramatically influence gap junctional communication among MNCs suggest a new set of functional dynamics that may exist in this system, as well as others for which the data are only beginning to become available. It could be said that the future has never held more exciting prospects.

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CHAPTER 2.1.3

Phenotypic and state-dependent expression of the electrical and morphological properties of oxytocin and vasopressin neurones

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Oxytocin and vasopressin secreting neurones of the hypothalamic supraoptic nucleus share many membrane characteristics and a roughly similar morphology. However, these two neurone types differ in the relative expression of some intrinsic and synaptic currents, and in the extent of their respective dendritic arbors. Spike depolarizing afterpotentials are present in both types, but more frequently give rise to prolonged burst discharges in vasopressin neurones. Oxytocin, but not vasopressin neurones, are characterized by a depolarization-activated, sustained outward rectifier which turns on near spike threshold, and which can produce prolonged spike frequency adaptation. When this sustained current is deactivated by small hyperpolarizing pulses, a rebound depolarization sufficient to evoke short spike trains follows the offset of these pulses. Both oxytocin and vasopressin neurones exhibit a transient outward rectification underlain by an I_a-type current. This transient rectifier delays spiking to depolarizing stimuli from a relatively hyperpolarized baseline, and is more prominent in vasopressin neurones. As a result, oxytocin neurones may be more reactive to depolarizing inputs. Both cell types receive glutamatergic, excitatory synaptic inputs and both possess *R,S*-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptor subtypes. The AMPA receptor channel on both cell types is characterized by a relatively high calcium permeability and voltage-dependent rectification, characteristic of a diminished presence of the GluR2 AMPA subunit. However, AMPA-mediated synaptic transients are larger, and decay faster, in oxytocin compared with vasopressin neurones, suggesting a potential difference for synaptic integration. The characteristics of NMDA-mediated synaptic transients are similar in oxytocin and vasopressin neurones, but some data suggest NMDA receptors may be less involved in the glutamatergic activation of oxytocin neurones. In both cell types, synaptic release of glutamate often coactivates AMPA and NMDA receptors. The dendritic morphology of oxytocin and vasopressin neurones in female rats differs from one another and exhibits considerable plasticity as a function of endocrine state. In virgin rats, oxytocin neurones have more dendritic branches and a greater total dendritic length compared with lactation, when the arbor is much less extensive. A complementary change occurs in vasopressin dendrites, which are more extensive during lactation. This reorganization suggests that oxytocin neurones may be more electronically compact during lactation. In addition, such dramatic shifts in overall dendritic length imply that significant gains and losses in either the total number of synapses, or in synaptic density, are incurred by both cell types as a function of reproductive state.

Introduction

Axonal release of the neurohypophysial hormones vasopressin (VP) and oxytocin (OT) into the blood stream is a direct function of the electrical activity of the parent cell bodies in the hypothalamus. Studies in vivo from rats have firmly established that antidromically identified OT and VP neurones of the supraoptic (SON) and paraventricular nucleus can be distinguished by firing patterns and responsiveness to certain peripheral stimuli (Poulain and Wakerley, 1982; Renaud and Bourque, 1991). These firing patterns, and the degree of synchrony among the neurones, dictate the amount and timing of hormone release. In addition, the reproductive state of the animal shapes the intrinsic (Stern and Armstrong, 1996) and synaptic

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(Brussaard et al., 1997) activity of these neurones, and exerts profound ultrastructural changes in cell-to-cell contacts which include both synaptic and neuronal-glial remodeling (Hatton, 1990; Theodosis and Poulain, 1993).

In the past few years, work in this laboratory has focused on: 1) determining whether unique membrane and transmitter receptor properties underlie differences in the firing patterns of OT and VP neurones; and 2) determining the degree of participation of OT and VP neurones in state-dependent plasticity. Due to mechanical instability of the highly vascularized ventral hypothalamus in vivo, addressing these issues has required in vitro intracellular recording techniques. While this approach has the advantage of affording superior recording conditions, the systematic physiological identification of OT and VP neurones, so carefully cultivated in vivo over the past 25 years using peripheral stimuli, is obviously impossible. However, using intracellular filling and immunochemical labelling, peptidergic identification is routinely accomplished from recordings in slices or hypothalamic explants (e.g., Yamashita et al., 1983; Cobbett et al., 1986; Erickson et al., 1990; Smith and Armstrong, 1993). This approach has garnered several differences between OT and VP neurones which could provide insight into their distinct functions.

Differentiating intrinsic properties of VP and OT neurones

Depolarizing afterpotentials

The phasic bursting activity characteristic of putative, active VP neurones in vivo had been observed to occur spontaneously in some of the early in vitro recordings from the SON and PVN (Hatton et al., 1978; Mason, 1980; Pittman et al., 1981; Armstrong and Sladek, 1982; Andrew and Dudek, 1983). A direct association of this activity with VP neurones was made by Yamashita et al. (1983) and Cobbett et al. (1986) by immunolabelling Lucifer Yellow-injected neurones. Andrew and Dudek (1983, 1984) identified an underlying depolarizing afterpotential (DAP) in phasic bursting neurones thought to drive the plateau potential maintaining burst discharge. There is little question that the DAP is critical for the characteristic burst discharges of VP neurones, but its incidence is not restricted to VP neurones (Erickson et al., 1993; Smith and Armstrong, 1993; Armstrong et al., 1994; Stern and Armstrong, 1996). Since only a few OT neurones exhibit phasic bursting activity in vivo (Poulain and Wakerley, 1982; Moos and Ingram, 1995) and in vitro (Armstrong et al., 1994), the DAP is most likely a necessary but not a sufficient condition for this activity. Nevertheless in both cell types an individual DAP can evoke a short, if not always a prolonged burst of activity. Whereas the mechanism of the DAP may be related to a T-type calcium current in guinea pig (Erickson et al., 1993), in rats it has been attributed both to an underlying, voltage-dependent mixed cationic conductance (Bourque, 1986) and to the deactivation of a potassium current (Li and Hatton, 1997). The DAP is strongly calcium-dependent (Bourque, 1986; Andrew, 1987) and in OT neurones may be suppressed by the endogenous calcium buffer, calbindin (Li et al., 1995).

Transient outward rectification

In slices or hypothalamic explants, a prominent feature of magnocellular neurosecretory neurones is a transient outward rectification (TOR) in response to depolarizing current from a relatively hyperpolarized (<-80 mV) membrane potential (Bourque, 1988) (Fig. 1). In both the SON (Armstrong and Stern, 1997) and PVN (Tasker and Dudek, 1991), the presence of a strong TOR successfully discriminates the magnocellular neurones from surrounding parvocellular types. Voltage clamp studies indicate that the TOR results from the activation of transient potassium current most like I_a (Bourque, 1988; Cobbett et al., 1989; Nagatomo et al., 1995; Li and Ferguson, 1996; Hlubek and Cobbett, 1997; Widmer et al., 1997) (Fig. 1). This current is rapidly activated subthreshold to action potentials and poised to strongly influence the firing of SON neurones (Randle et al., 1986; Renaud and Bourque, 1991).

Although OT and VP neurones show many similarities in their response to current injection (Erickson et al., 1993; Armstrong et al., 1994), in rats they

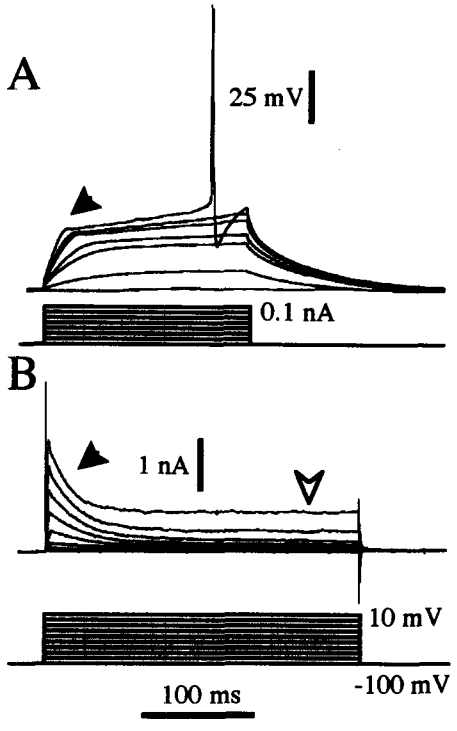

Fig. 1. Supraoptic neurones are characterized by a strong transient outward rectification (TOR). (A) Whole cell, current clamp recording from a SON neurone in a hypothalamic slice from a female rat. The neurone is held at -90 mV with current injection, then depolarized in steps to 0.1 nA. Note the prominent TOR (arrowhead) which is followed by a delayed, ramp depolarization. At the largest current injection, a spike is evoked toward the end of the ramp. (B) Whole cell, voltage clamp recording from a hypothalamic slice with TTX (0.5 μM) added to block voltage-gated sodium channels. The neurone was held at −100 mV and depolarized in 10 mV steps to +10 mV. Voltage-dependent transient (filled arrowhead) and sustained (empty arrowhead) outward currents are present. The transient current is an I_a-like current which underlies the TOR present in current clamp. Whole cell recordings were made with a potassium methylsulfate (130 mM) based internal solution.

differ significantly in the presence of outwardly rectifying currents (Stern and Armstrong, 1996; Fisher et al., 1997; Widmer et al., 1997). Unfortunately, the direction of this difference is under contention. In current clamp experiments from adult female rats, the TOR was found weaker at similar membrane potentials in OT compared with VP neurones (Stern and Armstrong, 1996). An analogous result was reported by Fisher et al. (1998), who found a lower peak I_a in dissociated OT neurones from adult rat SON using voltage clamp. In contrast, Widmer et al. (1997) found I_a virtually absent in VP neurones in dissociated neurones taken from rats of both sexes. Since the work of both Fisher et al. (1998) and Widmer et al. (1997) were done in dissociated neurones, the explanation that I_a resides solely on the dendrites of VP neurones, stripped off during dissociation, seems unlikely (Widmer et al., 1997). Further indication of a dichotomy of I_a distribution in the SON neurones is offered by O'Regan and Cobbett (1993), who found it absence in almost one-half of the dissociated rat SON neurones studied. Unfortunately, these neurones were not immunolabelled. In a recent study of potassium currents in dissociated guinea pig SON neurones, however, I_a type currents were ubiquitous (Hlubek and Cobbett, 1997).

Functionally, Fisher et al. (1998) found that the diminished expression of I_a is correlated with a shorter latency to spike activation from a hyperpolarized membrane potential for OT neurones, which could make them synaptically more excitable. Interestingly, the KV4.2 potassium channel subunit, associated with I_a type channels, is found near postsynaptic densities in the SON (Alonso and Widmer, 1997). Inhibition of I_a by angiotensin II (Nagatomo et al., 1995; Li and Ferguson, 1996), noradrenaline (Randle et al., 1986; Renaud and Bourque, 1991) and glutamate (working through metabotropic receptors; Schrader and Tasker, 1997) undoubtedly contributes to the excitatory effects of these compounds on SON and PVN neurones.

Sustained outward rectification

OT and VP neurones also differ in the presence of a sustained outward rectification (SOR) which is activated near spike threshold in ~95% of OT neurones in female (Stern and Armstrong, 1995) (Fig. 2) and male (Armstrong and Stern, 1998) rats. Unlike the TOR, the SOR has a slow onset, and remains active at depolarized potentials. From membrane potentials −50 mV or more positive, the

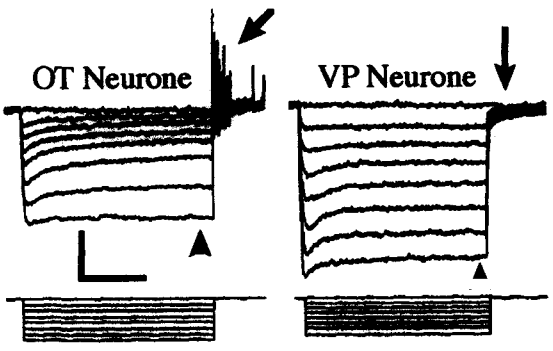

Fig. 2. Sharp electrode, current clamp recordings from immunocytochemically identified oxytocin (OT) and vasopressin (VP) neurones from a hypothalamic explant. In both cases, the membrane potential was held at −50 mV with current injection. Hyperpolarizing current pulses revealed a strong outward rectification from −50 to ~−70 mV in the OT neurone, but not in the VP neurone (arrowheads). In the OT but not the VP neurone, the offset of hyperpolarizing pulses was followed by a rebound depolarization of a few millivolts, reaching spike threshold (arrows). Vertical scale, 20 mV, 0.3 nA; horizontal scale, 0.5 ms. Each trace represents an average of two. Adapted from Stern and Armstrong (1995).

deactivation of the SOR with hyperpolarizing pulses produces a depolarizing sag and a rebound depolarization which are not due to the inward rectifier, I_h (Stern and Armstrong, 1997a). In contrast, the great majority of VP neurones lack the SOR and the rebound. Pharmacological and ion-substitution experiments confirm that the SOR is due largely to a potassium current (Stern and Armstrong, 1997a). This is not to argue that VP neurones do not have other outward potassium currents in addition to I_a, only that their behavior must be quite different from the SOR found in OT neurones. Indeed, slowly activating, sustained potassium currents have been described in SON and PVN neurones in a variety of preparations using voltage clamp (Bourque, 1988; Cobbett et al., 1989; Legendre and Poulain, 1992; O'Regan and Cobbett, 1993; Nagatomo et al., 1995; Li and Ferguson, 1996; Hlubek and Cobbett, 1997; Widmer et al., 1997). Although Widmer et al. (1997) report the presence of a delayed rectifier in virtually all SON neurones studied, a quantitative comparison of OT and VP neurones regarding the properties of these other potassium currents is currently lacking.

Functionally, the SOR can act to limit firing of OT neurones to strong, prolonged depolarizations (Stern and Armstrong, 1996). However, since the SOR is active near spike threshold, the rebound depolarization which follows its deactivation is sufficient to produce a brief burst of action potentials in a silent neurone, or a transient increase in firing rate if the neurone is already firing (Stern and Armstrong, 1996). It should be made clear that the SOR and rebound depolarization do not provide a mechanism for intrinsic, periodic bursting in OT neurones like that observed during lactation. However, modulation of the SOR with neurotransmitters, especially those which hyperpolarize the membrane, may be an effective means of redistributing action potentials to more clustered patterns when combined with an excitatory drive. Thus these properties may facilitate the synaptic activation of milk-ejection bursts during suckling. Interestingly, Moos (1995) reported that while application of γ-amino-butyric acid (GABA) onto OT neurones during suckling inhibited the background firing rate, it also decreased the delay to the next milk-ejection burst.

Oxytocin and vasopressin neurones differ in their complement of postsynaptic receptors

Peptides and other neuromodulators

Although the release of OT and VP can be evoked by many of the same stimuli, such as hyperosmolality, hypovolemia and hypotension, selective release and inhibition can be achieved. A good example is the preferential release of OT during labor and lactation (Poulain and Wakerley, 1982). Anatomical connections specific to OT or VP neurones probably underlie these differences (Armstrong, 1995). In addition, the ensemble of postsynaptic receptors for neurotransmitters and other neuroactive agents may distinguish the two cell types and contribute to differential modulation of hormone release. For example, histamine selectively activates VP neurones via H_1 receptors to produce both a relatively small, slow depolarization (Armstrong and Sladek, 1985; Li and Hatton, 1996;

Smith and Armstrong, 1996) and an enhancement of the DAP (Smith and Armstrong, 1993). In contrast, histamine may act via H_2 or some related receptor to hyperpolarize OT neurones (Yang and Hatton, 1994). However, the physiological circumstances under which these differences would be manifest during endogenous histamine release have yet to be revealed.

The two neurone types also differ regarding the peptide receptors they contain. Although direct immunochemical identification is lacking, opiates appear to inhibit OT, but not VP neurones, via μ-receptors in vitro (Pittman et al., 1980; Wakerley et al., 1983; Wuarin and Dudek, 1990). Autoreceptors for OT and VP have now been identified on the homotypic neurone and act to increase intracellular calcium (Dayanithi et al., 1996). This effect may mediate the changes in firing patterns attributed to these peptides on magnocellular neurones (Leng and Mason, 1982; Abe et al., 1983; Freund-Mercier and Richard, 1984; Inenaga and Yamashita, 1986; Yamashita et al., 1987; Lambert et al., 1993; Gouzènes et al., 1998). Regarding OT, there is strong evidence that locally released peptide facilitates the bursting activity of OT neurones during milk ejection (Freund-Mercier and Richard, 1984; Lambert et al., 1993). The effects that VP exerts in vivo are highly dependent upon the ongoing firing rate of the neurone such that the phasic bursting activity optimal for VP release is promoted (Gouzènes et al., 1998). Another example of a selectively acting substance is atrial natriuretic peptide, which inhibits VP neurones but has no reported effect on OT neurones (Standaert et al., 1987; Yamamoto et al., 1991). However, these effects may be mediated by presynaptic inhibition of glutamate release rather than by postsynaptic receptors (Richard and Bourque, 1996).

Amino acid transmitters

The majority of synaptic terminals contacting OT and VP neurones contain either glutamate or GABA (Decavel and Van den Pol, 1990, 1992; Meeker et al., 1993; Gies and Theodosis, 1994) and these transmitters are responsible for the great majority of the synaptic activity in the SON and PVN. The receptors mediating these actions are diverse, consisting of both ionotropic and metabotropic varieties, with several possibilities of modulation via varying subunit configurations. Physiological studies implicate both glutamatergic and GABAergic transmission in mediating hormone release. For example, OT release during lactation is heavily dependent upon activation of the glutamate subclass of receptors responsive to the agonist *R,S*-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) (Parker and Crowley, 1993), whereas the inhibition of VP neurones to hypertension is dependent upon $GABA_A$ receptor activation (Jhamandas and Renaud, 1987). Hyperosmotic stimulation not only directly depolarizes SON neurones (Mason, 1980), it activates excitatory inputs to the SON from the region of the organum vasculosum of the lamina terminalis (OVLT) and median preoptic nucleus which utilize glutamate (Richard and Bourque, 1995), some of which mediate osmotically induced VP release (Sladek et al., 1995).

Although both AMPA (Gribkoff and Dudek, 1990; Wuarin and Dudek, 1993) and *N*-methyl-D-aspartate (NMDA) type glutamate receptors (Gribkoff, 1991; Yang et al., 1994) can be synaptically activated on SON neurones in vitro, there is evidence that OT and VP neurones differ regarding these two subtypes. Nissen et al. (1994, 1995) found that putative OT neurones in male rats failed to respond to locally applied NMDA in vivo, whereas VP neurones were potently stimulated. Synaptic activation from stimulating the OVLT evoked a strong NMDA component in VP, but not in OT neurones in vitro (Yang et al., 1994). Support for a differential expression of NMDA receptors comes from Richardson and Wakerley (1997), who found that the firing rates of OT and VP neurones activated with locally applied glutamate were equally inhibited by AMPA receptor blockade, whereas NMDA antagonism produced a greater inhibition of VP neurones. In contrast, an ubiquitous activation of SON neurones with NMDA in vitro (Hu and Bourque, 1992; Hussy et al., 1997), including identified OT neurones (Jourdain et al., 1996) and in the lactating female rat (Moos et al., 1997), has been reported. Furthermore, NMDA antagonists alter synaptic inputs

from the lateral olfactory tract to both continuous and phasic cell types (Yang et al., 1996).

We have investigated the issue of glutamate receptor distribution in the two cell types in female rats using patch clamp recordings from slices of the SON (Stern and Armstrong, 1997b). Neurons were filled with biocytin and immunocytochemically labelled for VP- or OT-neurophysins as described previously (Smith and Armstrong, 1993; Stern and Armstrong, 1996). Synaptic activity was studied either as miniature excitatory postsynaptic currents (mEPSCs) or evoked EPSCs. Examination of either type of activity showed NMDA and AMPA components in both neurone types (Fig. 3). The AMPA response in all neurones was characterized by a non-linear, inwardly rectifying I/V relation (Fig. 3A), and experiments in dissociated neurones revealed a relatively high permeability for calcium. Both characteristics are thought to be associated with a lack of the GluR-2 subunit (Burnashev, 1996), but at present the immunocytochemical or hybridization data for the presence of this subunit in SON neurones are lacking. A difference between cell types was revealed when examining mEPSCs, where it was found that VP neurones have lower peak mEPSCs coupled with a longer decay time than those in OT neurones (Fig. 4). Such differences may lead to differences in synaptic integration for the two cell types. The underlying mechanisms responsible for these differences, which could include differences in glutamate reuptake, receptor desensitization, or receptor configuration, are at this point undetermined. Ginsberg et al. (1995) have found the GluR3 subunit more prevalent in OT compared with VP neurones, but it is difficult to relate this finding directly to the amplitude and kinetic differences we have observed. Most mEPSCs involve coactivated AMPA and NMDA-mediated components, making it likely that synaptic activation from some pathways at least, will recruit both synaptic forms. No difference was noted in the kinetics or amplitudes of NMDA mediated mEPSCs between OT and VP neurones.

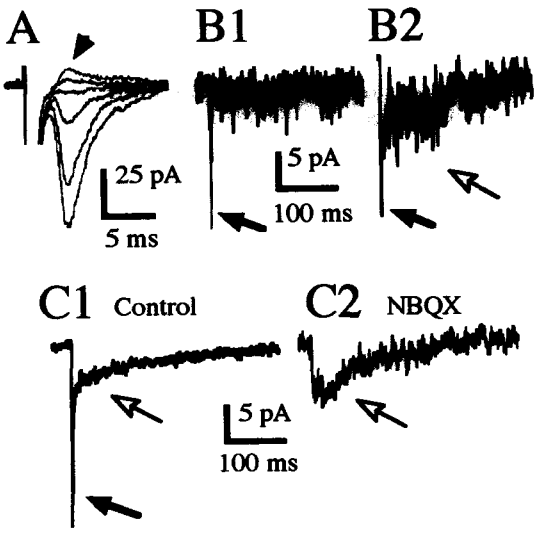

Fig. 3. Excitatory postsynaptic currents (EPSCs) in SON neurones recorded in hypothalamic slices from female rats. (A) AMPA-mediated EPSCs recorded in response to single shocks delivered dorsolateral to the SON in the presence of the NMDA antagonist, D,L-2-amino-5-phosphonopentanoic acid (±APV) (100 μM). The large inward current was strongly rectified at +20 and +40 mV (arrowhead), consistent with a diminished presence of the GluR2 AMPA receptor subunit. Each trace is an average of four. (B and C) Miniature EPSCs (mEPSCs) recorded in the presence of tetrodotoxin (TTX) (0.5 μM) at −50 mV. Extracellular magnesium was lowered to 20 μM to alleviate the voltage-dependent block of NMDA receptors. (B1) In normal media, mEPSCs were characterized as either single fast events (arrow) or more often as a fast event followed by a second, slower event (empty arrow, B2). The combined response suggests coactivation of both receptor types at a single synapse. (C) Averages of several hundred AMPA- and NMDA-mediated mEPSCs at −50 mV. (C1) In normal media, the average mEPSC shows both a fast (arrow) and slow (empty arrow) component. (C2) The fast component is blocked by the specific AMPA receptor antagonist, 6-nitro-7-sulphamoylbenzo(*f*)-quinoxaline-2,3-dione (NBQX) (10 μM), leaving only the slower, NMDA mediated component (empty arrow), which is blocked by APV (not shown). All recordings were made with a cesium gluconate (130 mM) based internal solution.

Oxytocin and vasopressin neurons have distinct dendritic topologies

Characterizing the synaptic actions resulting from the activation of particular receptors is but one part of the puzzle needed to understand synaptic integration. Extended dendritic trees place a

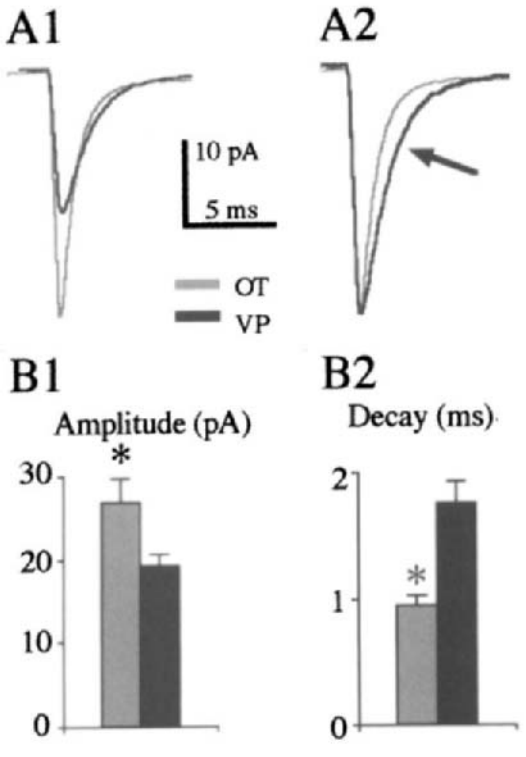

Fig. 4. Differences in AMPA-mediated mEPSCs between OT and VP neurones recorded in hypothalamic slices from female rats. All currents were recorded in the presence of TTX (0.5 μM) and the NMDA-receptor antagonist ±AP5 (100 μM), at −70 mV. The mEPSCs chosen for analysis exhibited rise times ≤0.4 ms in order to insure their more proximal location and thus reduce space clamp errors. (A) Averages of several hundred mEPSCs from a VP (black traces) and OT neurone (gray traces). Note the smaller amplitude of the mEPSC in the VP neurone. (B) The same two traces as in (A), except that the smaller mEPSC from the VP neurone has been scaled to the amplitude of the OT mEPSC. Note the slower decay of the VP mEPSC (arrow). (C) OT mEPSCs are significantly larger (left panel) and faster (right panel) than VP mEPSCs. Each histogram represents the mean (±SEM) of eight neurones. $*P < 0.01$.

degree of complexity on neuronal processing due nor only to electrotonic properties, but also because of the possibility of a non-uniform dispersion of transmitter receptors and ion channels. GABAergic (Decavel and Van den Pol, 1990; Gies and Theodosis, 1994) inputs synapse on both somatic and dendritic membranes of SON neurones and account for 30–50% of all synapses, similarly contacting both VP and OT neurones. Glutamatergic terminals account for ~20% of all synapses in the SON proper (Meeker et al., 1993; El Majdoubi et al., 1996) but may account for a larger percentage of terminals in the dendritic lamina of the SON (Meeker et al., 1993).

In female rats, there is considerable synaptic plasticity as a function of pregnancy and lactation, such that GABAergic (Gies and Theodosis, 1994) and glutamatergic (El Majdoubi et al., 1996) synapses account for higher percentages of the axosomatic synapses on OT, but not VP, neurones than in virgin animals. Since overall synaptic density appears not to change (Gies and Theodosis, 1994), the question arises as to whether axodendritic contacts are similarly modulated. Unfortunately, due to difficulties in identifying OT and VP dendrites in the electron microscope, the extent to which dendritic postsynaptic sites are involved in the differential innervation of VP and OT neurones is not well documented.

In previous Golgi and intracellular staining studies, SON neurones have been described as having a few, short and simply branching dendrites, but the cytoarchitecture of identified VP and OT neurones had not been examined (Armstrong, 1995). Because the interpretation of synaptic plasticity depends in part on target area, we filled identified SON neurones with Neurobiotin in explants from both virgin diestrous and lactating rats and reconstructed the dendritic trees in three dimensions (Stern and Armstrong, 1998). What we found was, to us, surprising. In diestrous females, OT neurones had on average two dendrites which gave rise to about eight total branches. In contrast, VP neurones showed on average only about two branches on the same number of dendrites. These changes were accompanied by differences in total dendritic length (Fig. 5). Total dendritic length and branching frequency decreased during lactation in OT cells, whereas for VP neurones, both parameters increased. The inverse relationship of OT and VP dendrites suggests that if unidentified processes were to be examined in both states, no change might be detected. This dynamic growth and shrinkage suggests that the reorganization of synapses needs

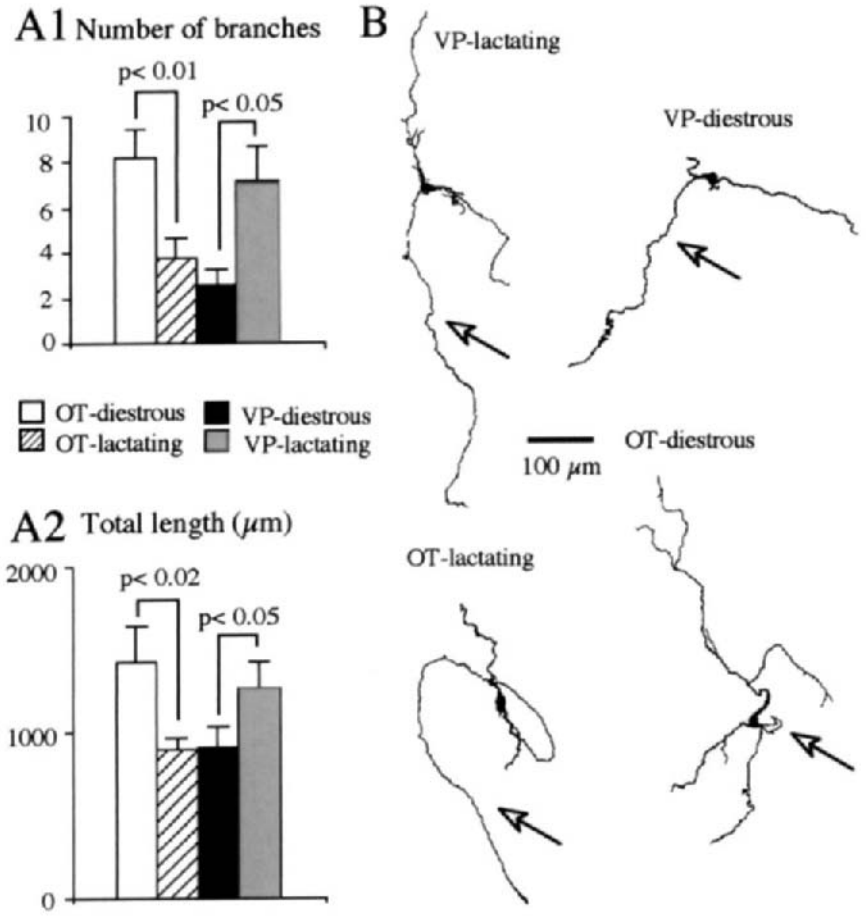

Fig. 5. The dendrites of VP and OT neurones are dynamically altered by endocrine state. (A1) The dendrites of OT neurones lose branches from virgin, diestrous females to lactation, whereas those of VP neurones gain branches. (A2) The loss of branches in OT neurones during lactation is associated with an overall reduction in dendritic length, whereas the increased branching of VP neurones results in a complementary increase in dendritic length. (B) Drawings of OT and VP neurones illustrating the dynamic changes in dendrites from virgin to lactating animals. In each drawing, the axon is indicated by the empty arrow. Modified from Stern and Armstrong (1998).

closer examination relative to the specific dendritic tree involved. Unless axodendritic synaptic density is markedly increased on OT neurones during lactation, we would predict a net loss of synapses on these neurones. Conversely, VP neurones may actually gain inputs during lactation without a change in density, or may decrease synaptic density by redistributing the same total number of inputs. At this time, it is unknown whether these changes are manifest during late pregnancy as are other ultrastructural modifications (Theodosis and Poulain, 1993), or depend upon active suckling.

Dendritic architecture strongly influences not only synaptic filtering (Rall, 1977), but could even shape firing properties, depending upon the distribution of ion channels along the dendritic arbor (Mainen and Sejnowski, 1996). The changes we have observed would suggest that OT neurones may be electrotonically more compact during lactation. The proper interpretation of this change will depend, among other things, on whether synaptic

density changes, and whether isolated or synchronous inputs are activated. In general, a pruned dendritic arbor will be one that is functionally less complicated, with fewer opportunities for synaptic integration, but one which could potentially provide some powerful inputs to the soma due to the shorter electrotonic length of the dendrite. An expanded tree may weaken remote, asynchronous inputs, but may allow for linear summation of synchronous inputs. Synaptic density and activity need to be characterized in these two cell types, under both conditions, before stronger conclusions can be drawn.

Discussion

In the past few years we have attempted to provide a baseline for the characterization of the membrane, morphological, and synaptic properties of identified OT and VP neurones. Although OT and VP neurones share many properties, they are electrophysiologically and morphologically distinct. In the future it will be crucial to provide identification of the two cell types to properly interpret both electrophysiological and anatomical experiments. We expect the difference in potassium currents between the two cell types, for example, to strongly condition the neurone's response to neurotransmitters. Furthermore, each type will adapt differently to imposed changes in firing. For example, I_a probably contributes to spike width in addition to modulating interspike interval in SON neurones. O'Regan and Cobbett (1993) and Jackson et al. (1991) showed that spike trains are sufficient to inactivate this current and promote spike broadening, the consequence of which is enhanced calcium influx. Thus, differences in I_a in the somatodendritic membrane of the two cell types may have consequences for the way in which OT and VP are released from dendrites, or on other calcium dependent events. If passed along to the terminals, such differences may contribute to differences in stimulus-secretion coupling for the two hormones (Bicknell, 1988). The prominent presence of a sustained, outwardly rectifying current in OT neurones appears to contribute to a delayed spike frequency adaptation in these neurones (Stern and Armstrong, 1996). This current appears strong enough at spike threshold that its modulation by transmitters should have a profound effect on firing rate. In addition, activation of this current will strongly affect electrotonus in these neurones, effectively lengthening the dendrite. In contrast, VP neurones may be more acutely modulated by transmitters targeting I_a. Activation or inhibition of either current may interact with the DAP's ability to produce prolonged bursts (Randle et al., 1986; Stern and Armstrong, 1996).

Since GABAergic transmission and its steroid modulation are strongly influenced by the reproductive state of female rats (Brussaard et al., 1997), it will be of great interest to determine the extent to which glutamatergic synapses are state-dependent. The AMPA receptor configuration appears different between the two neurone types. Of particular interest for future experiments will be determining the functional consequence of these differences in amplitude and decay, as well as the underlying subunit configuration responsible for the difference. The enhanced calcium permeability of the SON AMPA receptor means that subthreshold synaptic events could allow calcium influx to modulate other calcium dependent membrane properties as well as other synaptic inputs. This may depend on the proximity of calcium-dependent channels, for example, with the AMPA receptors, the cellular distribution of which is unknown at this date. However, at least one potassium channel protein, KV4.2, is found in dendritic membrane proximal to synapses (Alonso and Widmer, 1997). Although that particular channel is thought to represent a calcium-independent potassium current, a similar juxtaposition of AMPA receptors and calcium-dependent channels could lead to anatomically discrete interactions. Another possibility is that calcium influx through AMPA receptors may directly affect the colocalized NMDA receptor (McBain and Mayer, 1994).

Superficially, VP and OT neurones have a similar morphology, but our quantitative studies describe marked, state-dependent differences in dendritic architecture (Stern and Armstrong, 1998). Coupled with other studies demonstrating ultrastructural plasticity (Hatton, 1990; Theodosis and Poulain, 1993), receptor subunit changes (Brussaard et al., 1997) and regulatory changes in peptide expression

(Mezey and Kiss, 1991) during lactation, it is clear that the magnocellular neurosecretory system is anything but static in the adult female. Such dramatic changes signal both a network and a cellular adaptation to the demand for increased hormone release. Exactly how these changes interact to enhance OT release during lactation is a challenge for future research. Retraction and expansion of dendritic trees may be a mechanism to adjust total synaptic input, or a means to selectively enhance one population (i.e., those more proximal) at the expense of another. Perhaps during lactation sensory input from the suckling pups, the final pathway of which is unknown, is enhanced by a combination of electrotonic shortening and selective synaptic pruning. This determination will require identification of this specific input and its quantitative description in both virgin and lactating animals.

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CHAPTER 2.2.1

The magnocellular neurons of the hypothalamo-neurohypophyseal system display remarkable neuropeptidergic phenotypes leading to novel insights in neuronal cell biology

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For decades the magnocellular neurons of the hypothalamo-neurophypophyseal system (HNS), in which either vasopressin or oxytocin are produced and released into the bloodstream, have been playing a pivotal role in fundamental discoveries in the nervous system. The primary structure of vasopressin and oxytocin was the first of all neuropeptides to be published, i.e., in the 1950s by the Nobel prize laureate Du Vigneaud. Moreover, many trend-setting discoveries have their origin in the HNS, which abundantly expresses vasopressin and oxytocin, clearly displays its function and is relatively easily to manipulate. Examples are the phenomenon of coexpression of neuropeptides, patch-clamping of nerve endings, axonal transport of RNA, neuroglia interactions and the behavioral effects. An extraordinarily intriguing example is the homozygous Brattleboro rat, which lacks vasopressin by a germ-line mutation, and has disclosed many of the fundamental characteristics of peptidergic neurons, and neurons in general. In this chapter we will discuss a few of them, in particular the recent data on mutations in vasopressin RNA. It is to be expected that the HNS will retain its informative role in the next decades.

Introduction

The hypothalamo-neurohypophyseal system (HNS) consists of magnocellular neurons in which either the vasopressin (VP) or oxytocin (OT) precursor is synthesized, packaged and transported towards the terminals in the neural lobe, where the processed products (i.e., VP, neurophysin A and glycopeptide; OT and neurophysin B) are released into the bloodstream. Under conditions of extreme stimulation, such as lactation, VP and OT may be partially coexpressed by some neurons (Mezey and Kiss, 1991). The VP and OT genes are the major genes expressed in these cells. A large variety of neuropeptide genes (e.g., dynorphin, galanin) are expressed at a lower level. These precursors follow the same secretory pathway and are thought to be functionally involved in the fine-tuning of VP and OT release at the terminals and via the pituicytes within the neural lobe (Table 1; Boersma and Van Leeuwen, 1994). These neuropeptide precursors enter the regulated secretory pathway, where they are processed into their biologically active fragments by prohormone convertases such as PC1, PC2, PC5, carboxypeptidase E and the amidating enzyme peptidylglycine α-amidating monoxygenase (PAM) (e.g., Schäfer et al., 1992). These enzymes and the endocrine chaperone 7B2 are abundantly expressed within the magnocellular neurons of the HNS (Fig. 1).

In 1961 a naturally developed homozygous knock-out, i.e., the homozygous Brattleboro rat (di/di), was discovered (e.g., Valtin and Schroeder, 1964; Valtin and Schrier, 1997). The di/di rat

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suffers from severe hypothalamic diabetes insipidus caused by a single base deletion in exon B of the VP gene. This trait is autosomally recessive and inherited in a simple Mendelian manner. This deletion causes a −1 frameshift mutation and results in an entirely new C-terminus of the VP precursor. The changes of the C-terminus include the absence of five of the 14 cysteine residues, loss of a prohormone convertase cleavage site, the absence of the glycopeptide sequence and of a stop codon. As a result this aberrant precursor is arrested in the membranes of the endoplasmic reticulum and is unable to enter the secretory pathway (Fig. 1) (Schmale et al., 1989).

In the early 1980s when the complete VP and OT precursors were characterized, antibodies against specific fragments such as the glycoprotein moiety of VP became available. This was the start of a remarkable set of findings by a number of research teams (Mezey et al., 1986; Pow et al., 1992; Richards et al., 1985; Van Leeuwen et al., 1986), ultimately resulting in a new view on the etiology of Alzheimer's disease (Van Leeuwen et al., 1998).

Frameshift mutations

Using the human glycopeptide antiserum raised by the team of Dr. M. Chrétien (Clinical Research

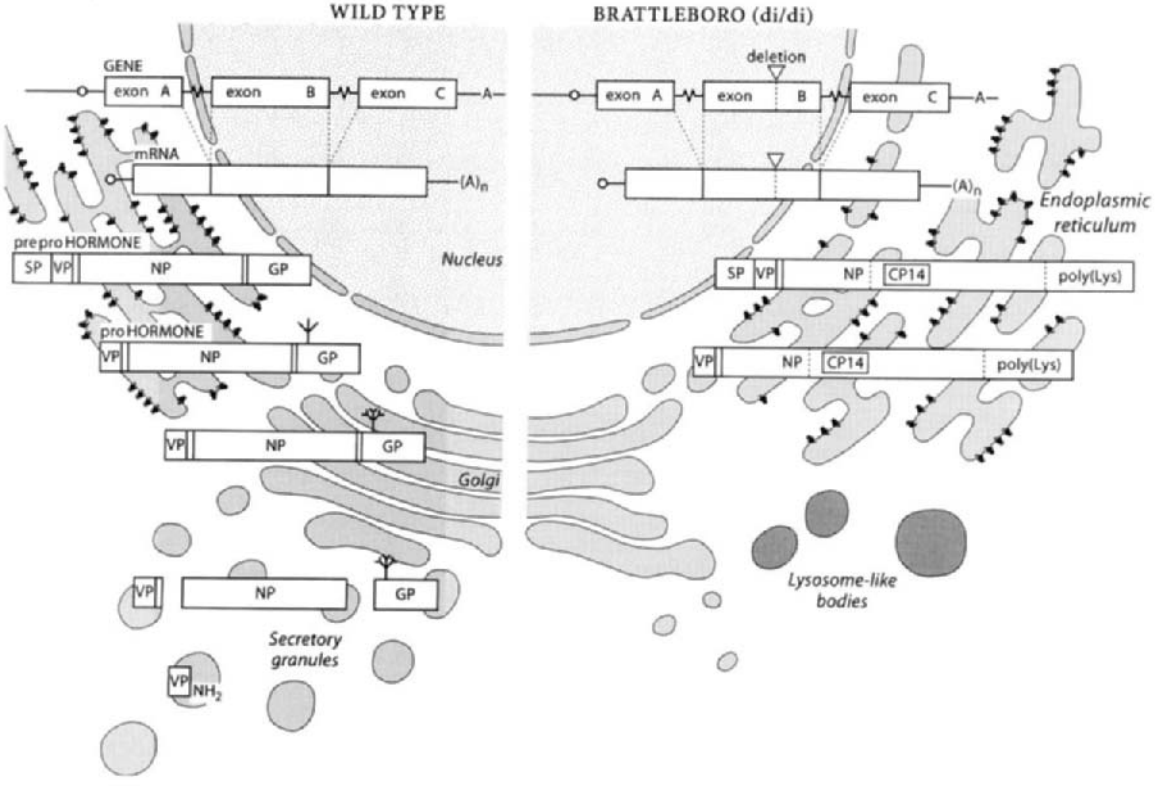

Fig. 1. Structural organization of the vasopressin (VP) genes from wild-type (left panel) and homozygous Brattleboro (di/di) (right panel) rats: their transcription, translation into a precursor protein that is posttranslationally processed (e.g., amidation) in magnocellular neurons. ER, endoplasmic reticulum; SP, signal peptide; VP; NP, neurophysin; GP, C-terminal glycopeptide of propressophysin; CP14, stretch of 14 amino acids predicted from the frame-shifted DNA sequence and used to generate mutant-specific antibodies. In the homozygous Brattleboro rat (di/di) the mutant precursor transport from the ER to the Golgi apparatus is disturbed, most probably by incorrect folding of the prohormone (see Schmale et al., 1989). However, a co-expressed peptide such as dynorphin is packaged in secretory granules that are much smaller (diameter 100 nm) than usual (Whitnall et al., 1985). (Picture adapted from Ivell et al., 1990).

Institute, Montreal) and a different one against sheep glycopeptide raised by Dr. D.G. Smyth (National Institute for Medical Research, Mill Hill, London, UK), a number of different teams revealed completely unexpected glycopeptide immunoreactivity in a subset of VP cells of the supraoptic and paraventricular nucleus of the di/di rat, which, according to the molecular biological predictions, should not exist (Mezey et al., 1986; Richards et al., 1985; Van Leeuwen et al., 1986). Since these antibodies were raised against highly purified VP-glycoprotein the results were received quite critically. Initially, findings were suspiciously explained by unexpected crossreactivity or a contaminating antibody. However, with an antibody against the rat synthetic glycopeptide fragment 22–39 (Seger and Burbach, 1987) similar results were obtained. Solitary cells that looked very hypertrophic displayed intense immunoreactivity, not only in the cell body, but also in the neurites running towards the neural lobe (Fig. 2A). The latter finding and the reaction to antisera

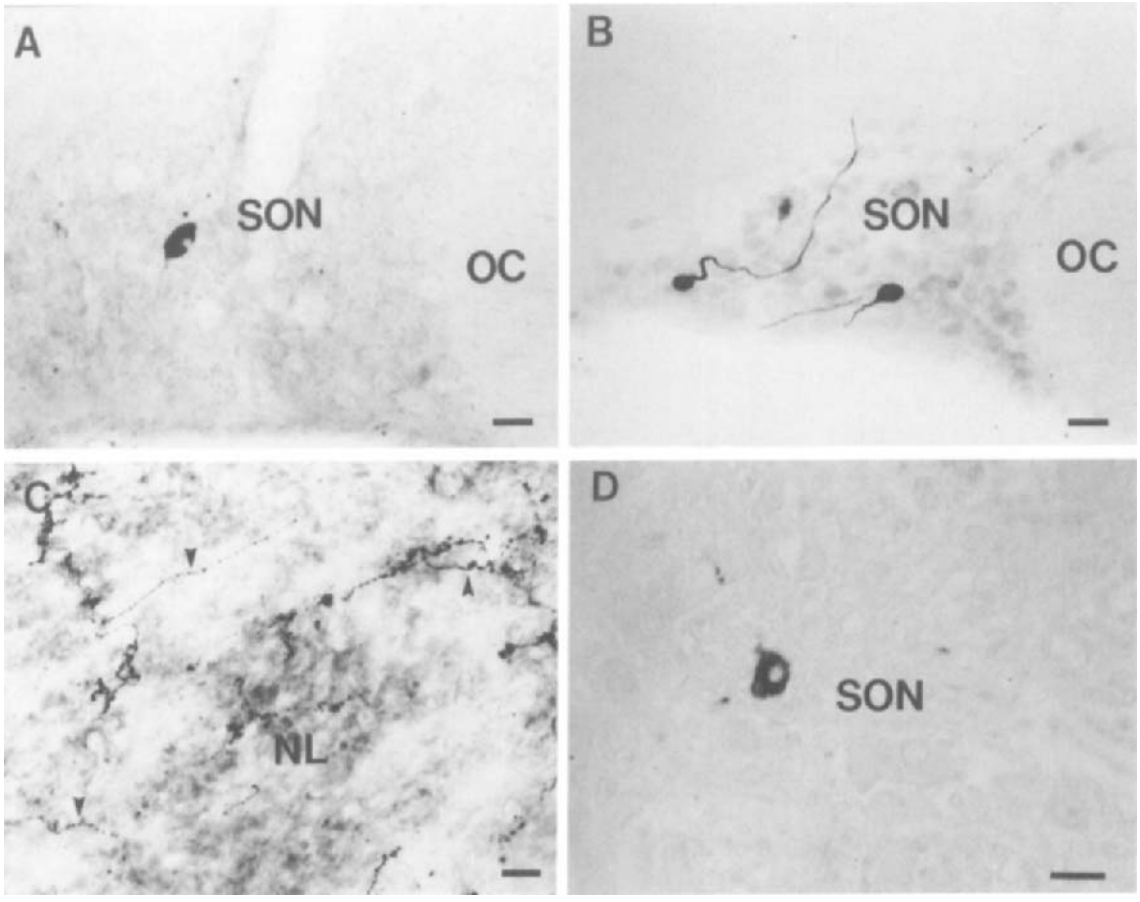

Fig. 2. Immunocytochemical localization of peptides expressed in the +1 reading frame (A) in the supraoptic nucleus of a homozygous Brattleboro rat using anti-neurophysin antiserum (#THR; for details, see Van Leeuwen et al., 1994). A solitary neuron shows cytoplasmatic staining around the nucleus. Bar = 50 μm. (B,C) In the supraoptic nucleus and the neural lobe (NL) of a wild-type rat using 474 antiserum (Evans et al., 1994). Note in B the fibers running towards the NL, where beaded fibers can be seen (arrowheads). Bar in B,C = 50 μm. (D) In the human supraoptic nucleus using HUVA antiserum (Evans et al., 1996). Note the intense cytoplasmatic immunoreactivity surrounding the nucleus. Bar = 25 μm. OC, optic chiasm; SON, supraoptic nucleus.

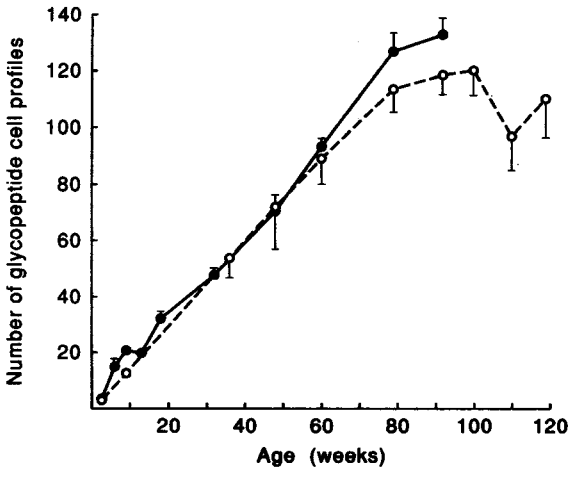

Fig. 3. Total number of GP-immunoreactive cell profiles in the hypothalamus of male (●) and female (○) homozygous Brattleboro rats at various ages. Note that the linear increase in number declines after 79 weeks of life. All animals suffered from a severe diabetes insipidus resulting in a very low osmolality of the urine (130–170 mOsm/kg H_2O) (from Van Leeuwen et al., 1994).

recognizing sequential epitopes in the VP precursor (Van Leeuwen et al., 1989) suggested that in some cells of di/di rats a normal VP precursor was synthesized that was also able to enter the secretory pathway. Indeed, it was shown that these cells and terminals contain neurosecretory granules with glycopeptide immunoreactivity (Pow et al., 1992; Sonnemans et al., 1996). A further remarkable finding was that these cells increased in number with age (Fig. 3). The latter finding supported a very old study by Valtin et al. (1965), who reported bioassayable VP in the neural lobe of a 21-month-old di/di rat. Furthermore, it was shown that these cells display a heterozygous phenotype (Van Leeuwen et al., 1989). Interestingly, glycopeptide immunoreactivity was not detected in the parvocellular VP neurons of various intra- and extrahypothalamic nuclei (e.g., suprachiasmatic nucleus) of di/di rats (Van Leeuwen et al., 1986, 1989).

In order to understand the events leading to apparently normal VP gene expression in these solitary cells with a revertant phenotype, in the early 1990s molecular biological studies were started aimed at the identification of the encoding cDNAs. Using mRNA of very aged di/di rats hypothalami (90 weeks) cDNAs were cloned that encoded VP precursors compatible with the observed immunoreactivities. In addition to the G deletion in exon B, these cDNAs carried a second dinucleotide deletion in exons B/C (Fig. 4). At two spots located in a GAGAG motif a GA deletion was found. These PCR based results were subsequently confirmed by stringent in situ hybridization detecting the GA deletion in solitary VP neurons. In the nearby located OT cells no indications were found for frameshift mutations, which can be partly explained by the absence of GAGAG motifs. However, other motifs may also give rise to alterations in mRNA (see below for human OT cells). The GA deletion downstream of the G deletion still results in an aberrant VP precursor with 13 and 22 amino acids different from wild type in the neurophysin moiety, whereas the glycoprotein part is of wild-type (Fig. 4). This partly corrected VP precursor is able to enter the secretory pathway. This may be possible since the aberrant stretch of

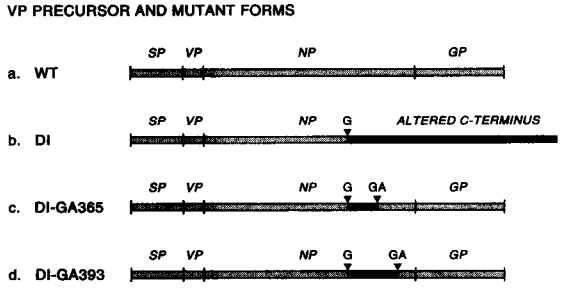

Fig. 4. Different forms of the vasopressin (VP) precursor in the wild-type (WT) and homozygous diabetes insipidus Brattleboro (di) rat. The VP gene encodes a precursor consisting of a signal peptide (SP), VP, VP-neurophysin and glycopeptide. (a) Wild-type rat VP precursor. (b) DI rat VP precursor. By a G deletion in the VP gene, the C-terminal part of neurophysin and glycopeptide is completely altered. (c,d) +1 di-VP precursors (DI-GA 365 + 393) expressed in reverted solitary magnocellular neurons. These precursors are encoded by VP transcripts with a GA deletion at two different sites downstream of the G deletion and result in a restored C-terminal NP and GP moiety. Compared with the wild-type VP precursor, a stretch of 13 (c) or 22 (d) amino acids is still mutated (with permission from Sonnemans et al., 1996).

13/22 amino acids in the neurophysin moiety is not critical for entering the secretory pathway (De Bree, 1996). A similar conclusion was drawn after transfection of AtT20 cells (Evans, 1995). Therefore, in postmitotic cells, as represented by the VP-producing supraoptic and paraventricular neurons, a mutation is generated after birth. Furthermore, it was shown that the increase in the number of these revertant cells can be modulated by VP substitution.

The next question that was addressed is whether transcript or genomic modifications (see below) only occur in di/di rats or also in wild-type VP cells. To answer this question proteins were predicted from the +1 reading frame of the VP gene based upon the knowledge obtained in di/di rats: a GA deletion. Indeed, using an antibody against such a theoretical +1 frameshift peptide, immunoreactivity was found in cells and fibers within the HNS of wild-type rats (Evans et al., 1994; Fig. 2B,C). The number of these cells displaying this type of +1 immunoreactivity was also age-related. Following the same strategy we also raised an antibody against a peptide predicted from a frameshift mutation (ΔGA) in human VP transcripts. Again solitary neurons were found in the paraventricular and supraoptic nucleus (Fig. 2D). Therefore the conclusion was drawn that dinucleotide deletions (and possibly also other frameshift mutations) may be a more general phenomenon in neurons with possible consequences for neuronal functioning and pathology. Indeed, in human OT cells, +1 frameshift proteins were also found. Their frequency, however, is much lower than that of VP cells. This may be related to the absence of a GAGAG motif, but indicates that other motifs may undergo a similar process of dinucleotide deletioning. Thus, other alterations in DNA or transcripts (insertions, deletions) also seem to be possible. For instance, in the di/di rat hypothalamus a low number of magnocellular neurons was found after the sections had been incubated with antiserum #474 (recognizing the +1 reading frame in wild-type rats; Evans et al., 1994; Van Leeuwen and Sonnemans, unpublished results). This immunoreactivity can only be explained by a base deletion or insertion different from a dinucleotide (ΔGA) deletion.

Are the mutations at transcript or genomic level?

The age-dependent increase in the number of VP cells with a revertant phenotype (Fig. 3) was originally interpreted as somatic mutations in the genes, occurring at an exceptionally high frequency (Evans et al., 1995; Finch and Goodman, 1997). The observations that argued against a transcript-related event were (a) during RNA-editing one would expect to find a similar degree of mutant VP expression in all hypothalamic VP neurons in di/di rats. However, frameshifted transcripts and precursors are found at high levels in a limited number of solitary cells, (b) the modifications lead to an irreversible phenotype, as shown by a constant increase in number with age (Fig. 3), and (c) the reverted solitary neurons of the di/di rat display both mutant and wild-type immunoreactivity within one cell (Van Leeuwen et al., 1989). These data strongly suggested the introduction of the mutation in one allele, thus involving DNA modifications.

The feeling that we were dealing with a genomic event was reinforced by the fact that we could manipulate the number of generated solitary revertant VP cells in di/di rats by long-term VP substitution (Evans et al., 1994). Long-term treatment with VP (40 weeks), which constantly normalized the water balance, resulted in a 25% decrease in the number of solitary neurons in di/di rats. A high metabolic rate (i.e., high transcriptional activity), as present in VP cells of the homozygous Brattleboro rat, was therefore correlated with an enhanced frequency of these solitary neurons. This observation was in agreement with data correlating increased transcriptional activity to chromosomal recombination (Nickoloff, 1992) or gene repair (Bohr and Wasserman, 1988).

However, we were subsequently unable to show a mutation at the genomic level, whereas in transcripts the mutation (ΔGA) could be readily determined. Using PCR amplified genomic vasopressin DNA, both screening with radiolabeled oligonucleotides (Evans, unpublished results) or after bacterial expression followed by immunoscreening similar to that reported by Evans et al. (1994), we failed to obtain positive clones even when solitary VP neurons of di/di rats were isolated and pooled

(Spence, unpublished results). Therefore the tentative conclusion was drawn that the events leading to revertant solitary neurons must be transcript-related. Of course a failure to show a positive result may be due to the sensitivity of the technique.

The idea that we indeed had to do with a transcript mutation was later confirmed in a different way. We reasoned that if a transcript mutation occurs in a GAGAG motif of di/di, wild-type and human VP, why would it not occur in other neuronal genes with GAGAG motifs, e.g., those involved in neurodegenerative diseases such as Alzheimer's disease (i.e., β-amyloid precursor protein (β-APP) and ubiquitin-B).

This indeed appeared to be the case (Van Leeuwen et al., 1998a,b). It is beyond the aim of this chapter to go into detail about this generalization, but again the question was raised whether the staining was due to a transcript or a genomic event. In the case of Alzheimer's disease several genes were studied and their proteins appeared to be colocalized, making a genomic event less likely. Moreover, similar to VP, immunoscreening of genomic PCR expression revealed no positive clones, whereas cDNA of transcripts were readily shown to contain the dinucleotide deletion, both in β-APP and in ubiquitin B. Here, again, the sensitivity of the method may have weakened the conclusion. Therefore, knowing where the mutations are located, an even more sensitive PCR strategy was developed detecting 10 plasmid copies out of a background of 500 ng genomic DNA (~160 000 copies). Since also in this case, where we finally analyzed 10 μg DNA of both genes (βAPP and Ubiquitin-B) of an Alzheimer and a Down syndrome patient, we failed to show any hybridization, we concluded that the events leading to a reverted phenotype in di/di rats, the aberrant phenotype in wild-type rat and human VP cells and mutant proteins in Alzheimer's disease must be due to transcript mutations.

Knowledge about the mechanism leading to dinucleotide deletions in and around GAGAG motifs is lacking. Transcript mutation may take place either co- or posttranscriptionally. Anyway it is different from substitutional RNA editing occurring in the nervous system (Simpson and Emeson, 1996).

Somatic non-homologous crossing over of VP and OT genes

The existence of yet another altered phenotype of magnocellular neurons was discovered by the group of Morris in the di/di rat (Pow et al., 1992). A small number of magnocellular neurons in di/di rats contains a very large accumulation of VP and C-terminal OT-neurophysin immunoreactivity in distended saccules of rough endoplasmic reticulum. The number of these cells is quite low: from 1–3 per hypothalamus in rats aged 0–6 months, with a maximum of 24 in 2-year-old animals and, interestingly, higher in females than in males and much higher in the supraoptic than in the paraventricular nucleus (Budd and Morris, 1997). For comparison, at the ages of 6 months and 2 years such rats contain respectively 35 and 110 of the solitary cells addressed above. In the saccules of the endoplasmic reticulum no immunoreactivity was detected for OT, VP-neurophysin and glycopeptide (Pow et al., 1992; Morris et al., 1995). PCR cloning resulted in the identification of 'hybrid' mRNAs, i.e., hybrid mRNA molecules with the 5' end of the VP sequence and the 3' end of the OT sequence. It was indicated that these transcripts result from somatic non-homologous crossing over of the VP and OT genes (Mohr et al., 1994). In most cases, the crossing-over occurs within the highly homologous exons B (Mohr et al., 1994). Interestingly, these mRNAs encode novel precursors with an appropriate reading frame. Indications for a similar phenomenon in wild-type rats were obtained by the identification of similar hybrid transcripts. However, so far no corresponding immunoreactivity has been detected in the hypothalamus of wild-type rats (Morris et al., 1995). As in the case of the +1 mutations, there is no direct evidence of genomic or transcript-related events underlying the alterations.

Cell biology of the co-expression of neuropeptides

Within VP and OT neurons a large variety of neuropeptides is co-expressed (Table 1). In the di/di rat the aberrant VP precursor is arrested in the membranes of the endoplasmic reticulum (Fig. 1).

Table 1
Co-existing peptides in VP and OT neurons and terminals in the rat hypothalamo-neurohypophyseal system

Neuropeptide	Co-existing with	Reference[a]
Dynorphin (Pro-enkephalin B)	VP	1,2,3,4
Galanin	VP	5,6
Neuropeptide Y (NPY)	VP	7,8
Angiotensin II (Ang II)	VP	9,10
Neuropeptide FF (NPFF)	VP	11,12
7B2	VP/OT	13
Enkephalins (Pro-enkephalin A)	VP/OT	1,14,15,16
Endothelin	VP/OT	17
Thyrotrophin-releasing factor (TRH)	OT	18
Corticotrophin-releasing hormone (CRH)	OT	19,20
Cholecystokinin	OT	15,19
Secretogranin II	VP	21
Nitric oxide synthase	VP/OT	22

[a] (1) Gaymann and Martin, 1987; (2) Watson et al., 1982; (3) Whitnall et al., 1983; (4) Whitnall et al., 1985; (5) Gai et al., 1990; (6) Skofitsch et al., 1989; (7) Larsen et al., 1992; (8) Kagotani et al., 1990; (9) Hoffman et al., 1982; (10) Imboden and Felix, 1991; (11) Majane and Yang, 1989; (12) Boersma et al., 1993; (13) Marcinkiewicz et al., 1985; (14) Adachi et al., 1985; (15) Martin et al., 1983; (16) Martin and Voigt, 1981; (17) Nakamura et al., 1993; (18) Tsuruo et al., 1988; (19) Bondy et al., 1989; (20) Burlet et al., 1983; (21) Ang et al., 1997; (22) Luckman et al., 1997.

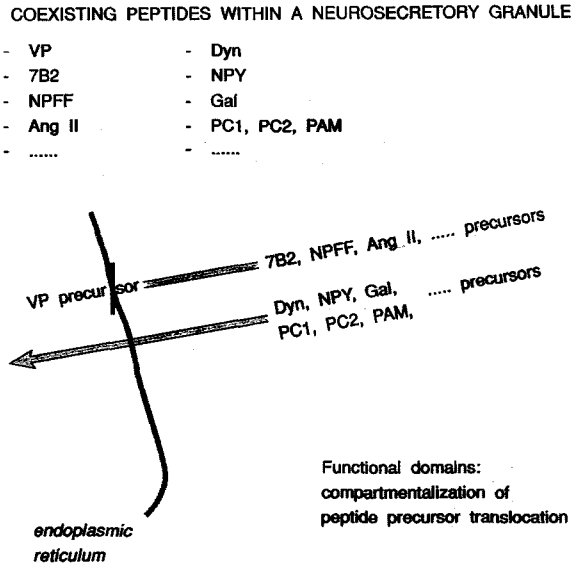

Fig. 5. Schematic representation of arrested vasopressin (VP) precursor in the membranes of the endoplasmic reticulum of a homozygous Brattleboro rat. The disturbed translocation of the VP precursor would also hinder the translocation of 7B2, neuropeptide FF (NPFF) and angiotensin II (Ang II). Since a number of other peptide precursors, such as dynorphin (DYN), neuropeptide Y (NPY), galanin (Gal), prohormone convertases (PC1 and PC2) and the amidating enzyme (PAM) enter the secretory pathway, their translocation is normal. In solitary neurons (Fig. 2A) only the VP precursor can be detected but also 7B2, NPFF and Ang II. Therefore it was postulated that at the membranes of the endoplasmic reticulum functional translocation domains exist.

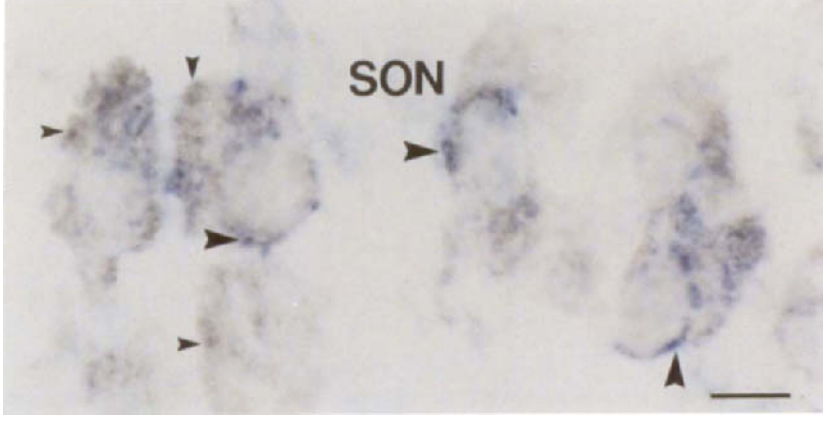

Fig. 6. Magnocellular vasopressin (VP) neurons in the rat supraoptic nucleus (SON) doubly labeled for VP (biotin-DAB, brown color, small arrowheads) and dynorphin (digoxigenin-alkaline phosphatase, blue color, large arrowheads) mRNA in cytoplasm. Note the different distribution of both transcripts and the negative nucleus. Magnification 1138×. Bar = 10 μm.

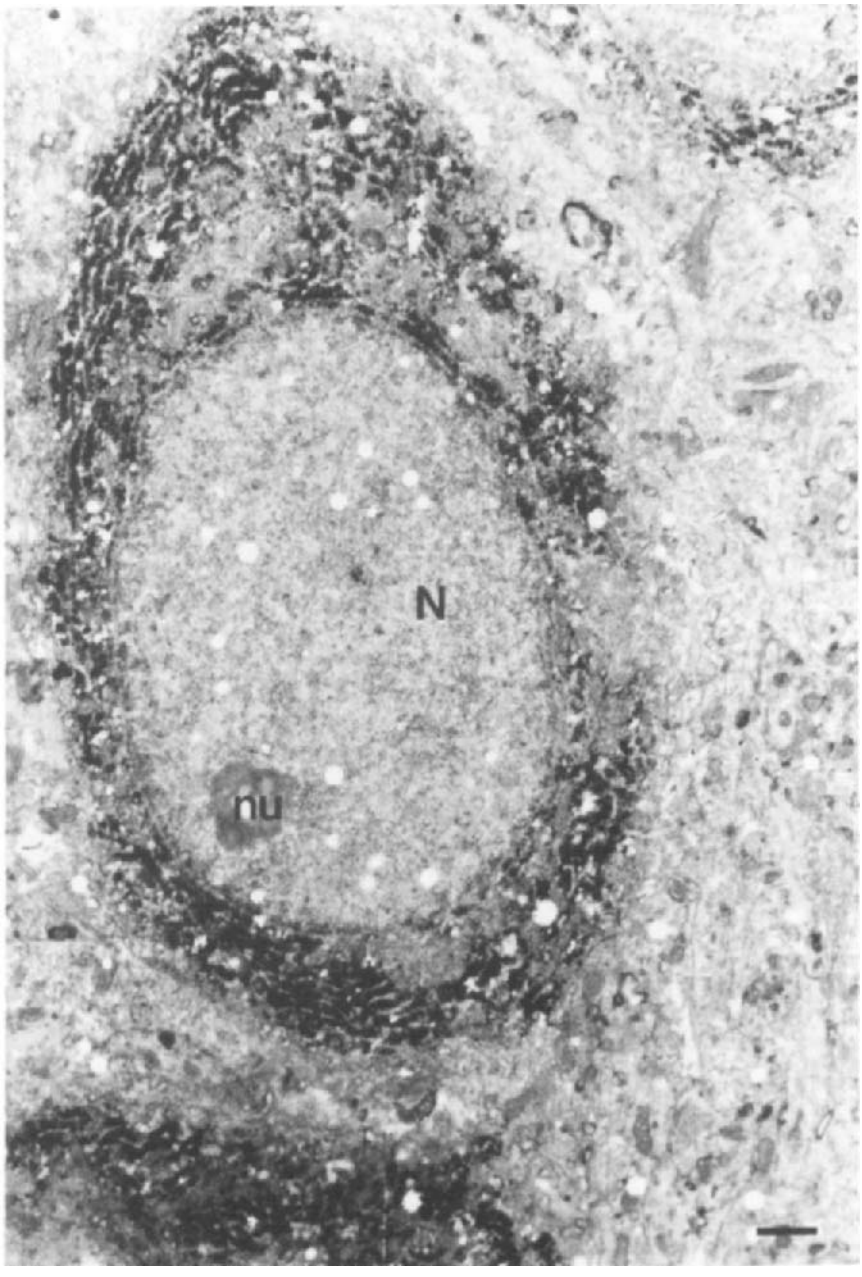

Fig. 7. Immunoelectron microscopic localization of vasopressin mRNA in rat magnocellular supraoptic nucleus neurons using biotin labeled cRNA. Note the localization of DAB-nickel deposits over the endoplasmic reticulum and the absence of the nucleus (N) and nucleolus (nu). Magnification 7800×. Bar = 1 μm.

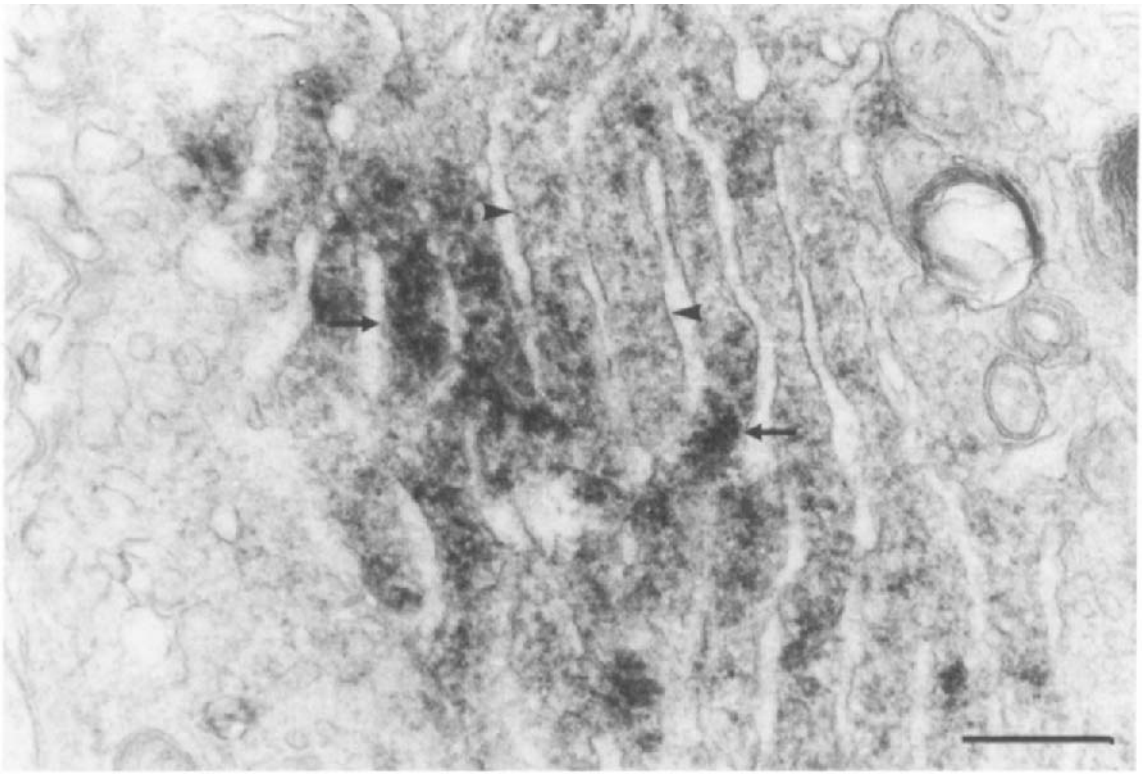

Fig. 8. A higher magnification shows both labeled (arrows) and unlabeled (arrowheads) cisternae of the endoplasmic reticulum. This indicates that specific domains of precursor peptide translocation may occur. Magnification 5000×. Bar = 0.5 μm.

A number of co-expressed neuropeptides nevertheless enters the secretory pathway (e.g., dynorphin) (Whitnall et al., 1985). Their expression seems to be unaffected. However, a number of neuropeptides (e.g., angiotensin II, neuropeptide FF and 7B2) are not detectable anymore in VP neurons of di/di rats (Van Leeuwen et al., 1991; Gabreëls et al., 1992; Boersma et al., 1993). It is remarkable that their immunoreactivity can again be detected in solitary cells with a revertant VP phenotype (see above, Fig. 2A). Their expression is thus correlated with a normal entry of VP into the secretory pathway. To explain these results we hypothesized that at the endoplasmic reticulum specific translocation domains are present (Fig. 5). Together with the VP precursor, the precursors of 7B2, angiotensin II and neuropeptide FF might be translocated at a site different from the one where dynorphin, galanin, NPY and other precursors enter the endoplasmic reticulum. Once the translocation of the VP precursor is disturbed, the intracellular trafficking of 7B2, angiotensin II and neuropeptide FF is also jammed ('the traffic jam hypothesis'; see also Finch and Goodman, 1997; Gabreëls, 1998). The question whether such specific domains indeed exist was addressed by double in situ hybridization at light and electronmicroscopic level. At the light microscopic level indeed a discrete localization of VP and dynorphin transcripts was found (Fig. 6), whereas at electron microscopic levels the endoplasmic reticulum was unevenly labeled by the VP probe (Figs. 7 and 8). These data were consistent with another study (Trembleau and Bloom, 1996) Unfortunately, technically it has so far not been possible to perform a reliable double labeling. Like the other organelles (such as the Golgi apparatus) the endoplasmic reticulum may be subdivided into functional domains. This indicates that the endoplasmic reticulum may

be involved in the fine-tuning of the differential synthesis of neuropeptide precursors. Similar suggestions for subdomains of the endoplasmic reticulum were obtained in completely different cellular systems (Okita et al., 1994; Okita and Rogers, 1996).

Conclusions

In conclusion, the HNS has provided neuroscientists with a number of very remarkable findings, as outlined above. The magnocellular neurons of the HNS have in many instances led neuroscientists to novel mechanisms and insights. The principles of neurosecretion defined in the past few decades were mainly based on studies in the HNS (e.g., Bargmann, 1949; Sachs and Takabatake, 1964). More recently, the above findings as well as the axonal transport of mRNA (Mohr et al., 1995) constitute examples of the power of the HNS to guide novel principles. The revertant VP cells of the di/di rats displayed the phenomenon of dinucleotide deletions in transcripts (Evans et al., 1994), which very recently allowed a generalization of this process in other gene transcripts involved in Alzheimer's neuropathology (Van Leeuwen et al., 1998a) and may be other age-related pathologies (Vogel, 1998). The process of the inaccurate conversion of genomic information into aberrant transcripts has recently been designated as molecular misreading (Van Leeuwen et al., 1998b). In the future too the extravagant cells of this system may be a starting point for new ideas concerning functioning, physiology and pathology of neuronal systems.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 2.2.2

Biochemistry of vasopressin fragments☆

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Vasopressin (VP) undergoes a step-wise aminopeptidase conversion process in the brain, leading to accumulation of several metabolites. Some of these metabolites, in particular [pGlu^4,Cyt^6]VP 4–9 and 4–8, show behavioral effects comparable to VP, but are more potent and selective than VP. Most data favor the existence of a separate receptor for the VP metabolites distinct of the classical VP and oxytocin receptors, although its identity has remained obscure thus far. The characterization of this receptor is a major challenge to understand how the brain VP system generates and regulates divers central functions.

Introduction

For over 20 years it is has been known that behavioral activities are not only exerted by VP as such but also by shorter peptides derived from the VP sequence. The prototype is DGAVP or DGLVP, the des-Gly-NH_2^9 forms of VP or [Lys^8]VP, VP 1–8 and LVP 1–8, respectively (De Wied, 1977; De Wied et al., 1993). In the 1980s a series of peptides generated from VP by brain enzymes were identified and found to be centrally active. These peptides may be products of extracellular metabolism of VP in the brain. Like DGAVP, these peptides do not seem to act through the now known VP receptor subtypes. This chapter reviews the biochemistry of VP metabolites and discusses ongoing studies towards the identification of VP metabolite receptors.

Processing of VP by brain enzymes

The first experiments on the conversion of vasopressin date from 1935 (Heller and Urban, 1935), and were performed from the viewpoint that enzymes degrade and inactivate the hormone. Along this line, Hooper and coworkers investigated the enzymatic 'loss of biological activity' of VP by brain extracts in the 1960s (Hooper, 1963). The first metabolites were identified by Pliška et al. (1971a,b). In the 1970s, the notion that VP and OT might serve as precursors for biologically active entities (Walter, 1972, 1974; De Wied, 1977; De Wied and Bohus, 1978) stimulated further work on the processing of VP by brain enzymes aiming to identify VP fragments, to elucidate their biological actions, and to characterize the enzymes involved in their formation.

Based on the concept that VP encloses multiple sites of central activity (De Wied and Bohus, 1978; De Wied, 1980; Burbach and De Wied, 1981), the identification of metabolites of VP generated during metabolism of the peptide by brain peptidases was undertaken. Reasoning that in situ formation of active fragments might occur in or near the sites of VP storage or release in order to allow the metabolites to exert a biological action, synaptic membranes were initially investigated for their capacity to convert VP.

Exposure of VP to brain membranes results in accumulation of a number of peptides that have

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been isolated by high-pressure liquid chromatography (HPLC) and chemically characterized (Burbach and Lebouille, 1983; Burbach et al., 1983a,b). In these experiments the peptides [Cyt^6]VP 2–9, [Cyt^6]VP 3–9, [pGlu^4,Cyt^6]VP 4–9, [Cyt^6]VP 4–9 and [Cyt^6]VP 5–9 were identified (Burbach, 1986, 1987). The structure of these metabolites and the events in their formation are shown in Fig. 1. All metabolites have the intact amidated C-terminus of VP. A structural feature of in vitro formed metabolites is the conserved disulfide bridge leaving Cys^1 linked to Cys^6. These fragments are asymmetric disulfides, which are stable under the in vitro conditions. However, in vivo the Cys^1 residue is rapidly lost due to reduction. Another characteristic of VP metabolism in vitro is the formation of the pyroGlu residue in [pGlu^4,Cyt^6]VP 4–9. This residue serves as a block for most aminopeptidases and can account therefore for the accumulation of [pGlu^4,Cyt^6]VP 4–9 during prolonged exposure of VP to brain membranes (Burbach et al., 1983a). PyroGlu formation can occur spontaneously, in particular under acidic condition.

The mechanism by which the main metabolites are generated in vitro has been corroborated in time-course experiments with the aid of ^{14}C-labelled VP preparations (Burbach and Lebouille, 1983). [^{14}C-GlyNH$_2^9$]VP incubated with a synaptic membrane suspension is converted stepwise. [Cyt^6]VP 2–9 and [Cyt^6]VP 3–9 appear sequentially as transient metabolites. The accumulation of [pGlu^4,Cyt^6]VP 4–9 follows later, while [Cyt^6]VP 4–9 and [Cyt^6]VP 5–9 remain low under these conditions. The pathway of conversion is typical for an aminopeptidase-like mechanism. A similar mechanism has been found for the conversion of OT and vasotocin (Burbach and Lebouille, 1983; Wang et al., 1983, 1985; Burbach, 1986).

In these in vitro experiments and one by Pliška et al. (1971a,b) there was very little cleavage in the C-terminus of VP. C-terminal cleavages accounted for approximately 10% of the total conversion of VP in brain membranes (Burbach and Lebouille, 1983). In vitro experiments of Marks et al. (1973) demonstrated that C-terminal cleavages were predominant in a soluble fraction of brain tissue. In all experiments, the cleavages in the C-terminus of VP resulted in formation of VP 1–7 as the main product with no or small amounts of VP 1–8 (DGAVP).

VP metabolites in vivo

The importance of VP processing at the N-terminus in vivo and the involvement of aminopeptidase

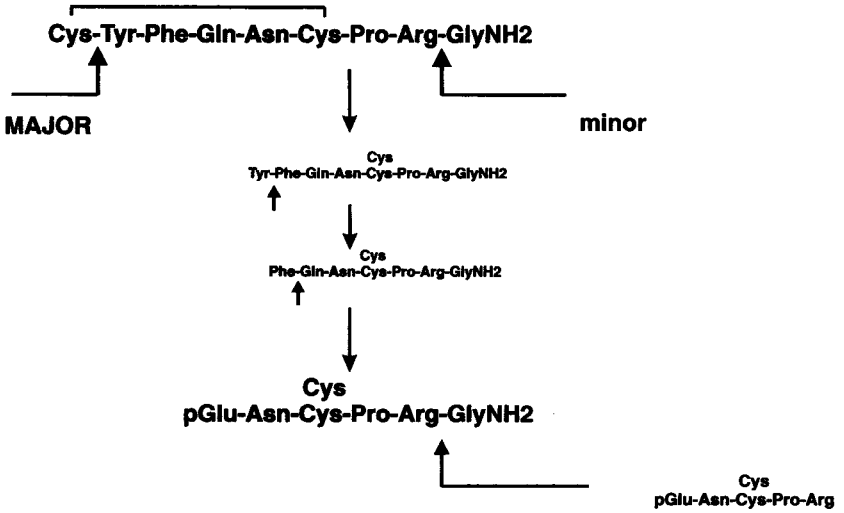

Fig. 1. Step-wise metabolism of VP in the brain. The major route involves the action of aminopeptidase activity and the accumulation of [pGlu^4,Cyt^6]VP 4–9 as relatively stable intermediate. C-terminal cleavages have a minor contribution.

activity has been demonstrated in biochemical and pharmacological studies. Firstly, C-terminal VP metabolites are present in brain tissue, whereas the peptide VP 1–8 is absent (Burbach et al., 1984b; Wang et al., 1985; Burbach and Liu, 1989). Secondly, the metabolites of [^{3}H-Phe^3]VP microinjected into the brain are mainly C-terminal VP fragments, with some VP 1–7 (Stark et al., 1989). Thirdly, the aminopeptidase inhibitor amastatin prolongs a central effect of VP in mice, but not the effect of N-terminally protected VP analogues.

The endogenous presence of VP fragments in the brain has been detected in experiments using immunological and chromatographic techniques (Burbach et al., 1984b; Wang et al., 1986; Burbach and Liu, 1989). Extracts of rat brain areas contain several components that cross-react in a radioimmunoassay system recognizing C-terminal VP fragments as small as [Cyt^6]VP 5–9. HPLC separation of immunoreactive components showed that some of them co-migrated with the in vitro identified VP metabolites such as [Cyt^6]VP 2–9, [Cyt^6]VP 3–9, [Cyt^6]VP 4–9, [Cyt^6]VP 5–9, [pGlu^4,Cyt^6]VP 4–9. Others co-eluted similar to reduced forms of these peptides. In these experiments N-terminal VP fragments like VP 1–8 were not found in extracts of brain and pituitary tissues (Burbach et al., 1984b; Wang et al., 1986). It should be realized that in these studies identity has only been based on immunoreactivity combined with chromatographic characteristics. Presently available powerful analytical tools like matrix assisted laser description mass spectrometry (MALSI-MS) are able to identify sets of peptides in a single cell (Jimnez et al., 1997) and allows revision of the endogenous VP metabolites, both qualitatively and quantitatively.

As expected for products of extracellular processing, the levels of VP metabolites in different brain regions parallels that of VP itself. However, in comparison to the content of intact VP, the putative VP fragments are most abundant in extrahypothalamic brain regions. In the hippocampus, amygdala, septum, thalamus, and medulla oblongata, the forms which chromatographically correspond to forms of 4–9 and 5–9 fragments of VP can account for up to 30% of the VP content (Burbach et al., 1984b; Burbach, 1986; Wang et al., 1986). Differences in amounts of the various components are seen. The proportion of VP metabolites is much less in the hypothalamus and are virtually absent in the pituitary gland. This observation seems biologically relevant since VP metabolites have central actions and are inactive in the periphery.

Part of the route of VP metabolism in vitro has been delineated by administering VP locally in the brain. Radiolabeled VP ([^{3}H-Phe^3]VP) administered by microinjection into the hippocampus or the amygdala is converted into C-terminal fragments (Stark et al., 1989). The conversion is very rapid: the half-life of injected VP is less than 1 min. The C-terminal fragments appear transiently. The profile of metabolites indicate that after the initial cleavage of VP (between Cys^1 and Tyr^2), the Cys^1 residue is rapidly lost due to reduction (Fig. 1). Consequently, the metabolites are [Cyt^6]VP 2–9, VP 2–9 and VP 3–9. Small amounts of VP 1–7 are also formed, but VP 1–8 has not been found (Stark et al., 1989). Amastatin co-injected in high doses reduces the conversion rate of VP resulting in a half-life of about 2 min. Intracerebroventricular injection of [^{3}H-Phe^3]VP also resulted in rapid conversion and release of ^{3}H-Phe, indicating a similar metabolic conversion of VP. Little accumulation of the labeled intermediate [Cyt^6]VP 2–9 was found (Stark, Burbach and De Wied, unpublished).

Taken together, the available data so far suggest that C-terminal VP fragments exist in the brain and that they are products of aminopeptidase action on VP in vivo. This indicates that the in vitro delineated metabolic steps are operative in situ as well. The data indicate the aminopeptidase conversion as the major metabolic route of extracellular VP degradation. C-terminal cleavages are minor (Fig. 1). The levels of C-terminal VP fragments are almost one order of magnitude lower than VP itself, suggesting that they may be short-living metabolites of VP. They are possibly generated transiently after release of VP from central sites (Fig. 2). This may relate to the hypothesis that VP metabolites are preferentially generated during activation of the VP systems, for instance during adaptation to novel environments (De Wied et al., 1987). Studies indicate that exogenously administered VP to the brain, i.c.v. or locally, also undergoes metabolism through aminopeptidase action and formation of active metabolites. Thus, a poten-

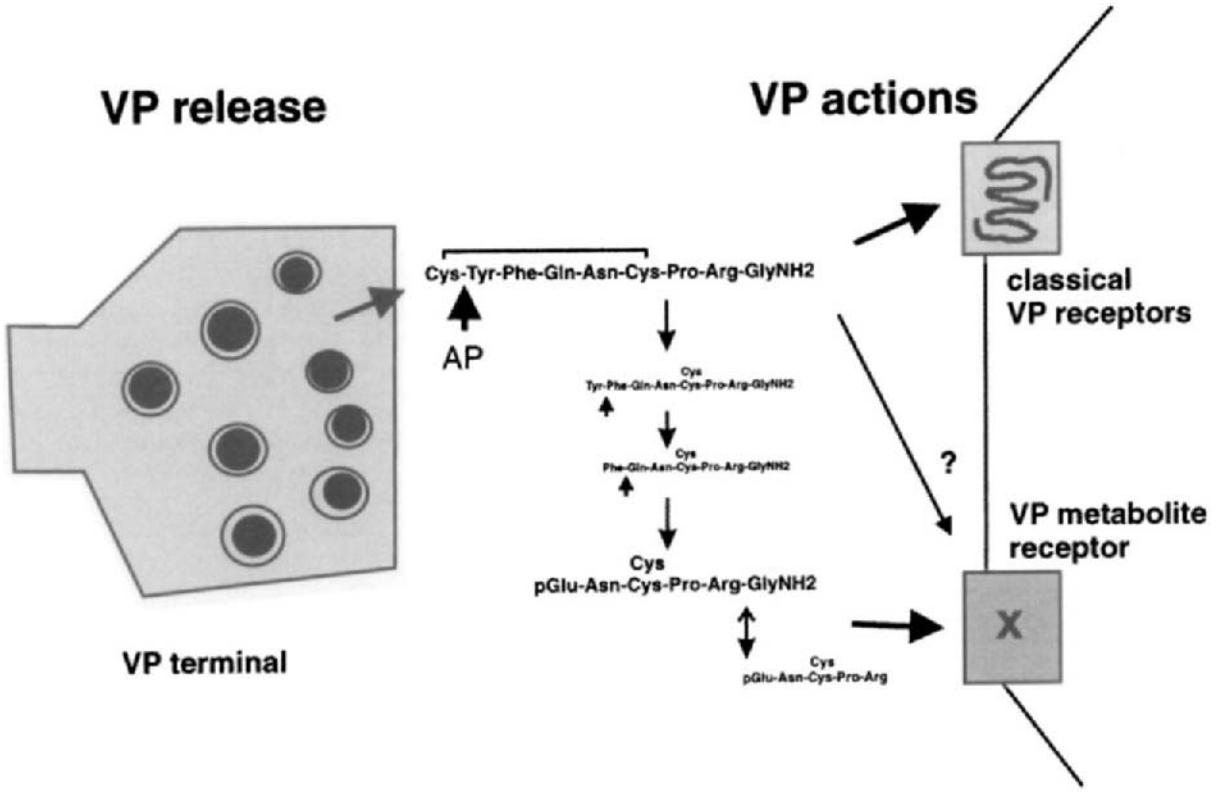

Fig. 2. Hypothetical events in the central actions of VP following release. VP can directly act on the classical VP/OT receptors (V_{1a}, V_{1b}, V_2, and OT-R). VP released from VP neurons also becomes subject to metabolic conversion by extracellular enzymes, mainly involving aminopeptidase activity. Intermediate metabolites act through independent receptors of unknown identity. An unanswered key question is if VP needs to be metabolized in order to activate this receptor, or may also activate this receptor directly.

tial contribution of these metabolites to the spectrum of central effects of VP is reminiscent to VP administration. However, it remains to be established to what extent they contribute to the actions of VP in the brain. For this reason it is essential to know the pharmacology of VP metabolites in detail, for which the identification of their receptor(s) is required (see below).

VP-converting aminopeptidase activity in the brain

The aminopeptidase activity responsible for processing of VP has tentatively been termed 'vasopressin-converting aminopeptidase activity' (VP-AP activity), but is by no means meant to suggest that an enzyme with exclusive specificity for VP exists (Burbach, 1987; Burbach and Liu, 1989). Characteristic for its action is the cleavage of the Cys^1-Tyr^2 bond in VP. This is the initial and rate-limiting step in the conversion cascade (Fig. 1). It results in opening of the N-terminal ring portion and generates the first linear metabolite [Cyt^6]VP 2–9. This linear peptide then becomes a substrate for various other aminopeptidases present in tissues. VP-AP activity resembles in proteolytic mechanism 'plasma oxytocinase', an aminopeptidase-like enzyme present in the circulation of pregnant women (Ferrier and Branda, 1966; Sjöholm and Yman, 1967). Overall properties of VP-AP activity in the brain could be established using an in vitro assay (Burbach, 1987; Burbach and Liu,

1989), but the enzyme has not been purified based on its action on VP.

VP-AP activity of brain membranes requires a thiol group and metal ion for full activity. Para-chloro-mercuribenzoate (OCMB), Hg^{2+} and EDTA were effective inhibitors of the VP-AP activity. The enzyme activity is dependent on a metal ion: there is inhibition by the metal chelator EDTA and full recovery of the EDTA-treated enzyme activity by 10^5M Zn^{2+} (Burbach, 1987). VP-AP in brain membranes has a pH optimum of 7.0 and a K_m of 12×10^{-6} M. It is present with highest specific activity in so-called 'P_3-membranes', an undefined membrane fraction of the brain and in synaptosomal and microsomal membranes (Burbach, 1987). VP-AP activity has also been detected in soluble fractions of the brain. All brain areas contain VP-AP activity with highest concentration in the preoptic area, cerebellum and bulbus olfactorius. Thus, VP-AP activity is rather a general cellular component of brain tissue than an enzyme strictly linked to the central VP systems. Properties of brain VP-AP activity related substrate specificity have been investigated by determining the inhibition of VP-AP activity by a number of peptides. Several VP analogues and VP fragments inhibited VP-AP action on [^{3}H]VP (unpublished). Several substitutions in VP did not change the inhibiting potency like Lys^8 and Leu^4. Other substitutions resulted in a moderate decrease in inhibiting potency, like Ala^2, des-amino-Cys^1, which flank the peptide bond cleaved by the enzyme. A clearly enhanced potency was found for VP 1–8, while the potency of VP 1–7 and [pGlu^4,Cyt^6]VP 4–9 was greatly reduced. Several peptides unrelated to VP also displayed considerable inhibition, in particular angiotensin I, somatostatin and porcine ACTH 1–39. The data suggest some structural preference of VP-AP activity, but meanwhile indicate a broad substrate specificity beyond VP, OT and VT.

Physiological situations in which VP-AP activity changes in relation to VP levels have been observed in preliminary studies to investigate whether there is a role of VP-AP activity in the control of levels of VP in the brain. In those studies, changes in VP-AP activity and VP levels in the pineal gland were determined in a physiological context, i.e. the spontaneous diurnal and seasonal variations in the gland. There is a rapid drop in pineal VP levels by about 35% at the onset of light, which lasts during the light period (Liu and Burbach, 1989a). VP-AP activity rose by about 30% at the onset of light. This rise was rapid and short-lasting (about 3 h). The same changes in peptide level and peptidase activity occurred at the same time point when the switch from dark to light was omitted, indicating circadian nature of the variations. Remarkably, a change in pineal VP level and VP-AP activity was also found to occur each year. In the first week of August pineal VP levels rise sharply by about 5–10-fold (Liu and Burbach, 1987). Shortly before this period, pineal VP-AP activity becomes about 25% lower than in the middle of July (Liu and Burbach, 1989b) and rises again in September. Other areas than the pineal gland do not display the diurnal and seasonal variations in VP level and VP-AP activity. The observations are suggestive of a relationship between VP-AP activity and VP level, in which increased or decreased enzyme activity causes VP levels to lower or elevate, respectively. This could be accompanied with changes in levels of endogenous VP fragments.

Receptors for metabolites of vasopressin and oxytocin

A number of pharmacological studies have demonstrated that VP metabolites are physiologically active peptides in the central nervous system. Many of these studies established that VP metabolites are involved in learning and memory processes similar to VP (Burbach et al., 1983a; De Wied et al., 1984; Dietrich and Allen, 1997; Vawter et al., 1997), although they display central activities at other levels as well (see also Reijmers et al., this volume). Electrophysiological studies showed that VP 4–8/4–9 caused a long-lasting enhancement of glutamate-mediated transmission in the lateral septum and ventral hippocampus, and that it decreased the threshold of hippocampal and lateral septal neurons to synaptic excitatory input (Urban and Killian, 1990; Rong et al., 1993). [pGlu^4,-Cyt^6]VP 4–9 or 4–8, indicated as VP 4–8/4–9 below, could also induce the activation of c-fos and c-src in hippocampal neurons after i.c.v.

administration (Giri et al., 1990; Gu and Du, 1991). VP 4–8 can affect the phosphorylation of the neural phosphoprotein B50/GAP43 (Chen et al., 1993). Furthermore, VP 4–9 was able to stimulate acetylchloline release in the hippocampus of freely moving rats (Maegawa et al., 1992) and VP 4–8 enhanced the expression of nerve growth factor in the hippocampus and cortex of the rat (Zhou et al., 1995).

A question that remains unsolved is whether the effects of VP 4–8/4–9 are mediated via established VP/OT receptors or via as yet unidentified receptors specific for these metabolites. To date four classical vasopressin/oxytocin receptors (the V_{1a}, V_{1b}, V_2 and OT receptors) are identified and each is a member of the seven transmembrane G protein-coupled receptor family (for review, see Burbach et al., 1995; Lolait et al., 1995). Antagonists for the V_{1a} and OT receptors were able to block behavioral effects of VP 4–8, suggesting that the actions may be mediated by the classical or very similar receptors (De Wied et al., 1991), However, several lines of evidence suggest the existence of a different receptor that mediates effects of VP metabolites than the classical VP/OT receptors.

Ligand binding studies have demonstrated that VP 4–8 and VP 4–9 are not able to displace labeled VP from the V_{1a} and V_2 receptors (unpublished) and do not display intrinsic activity on any VP receptor (Fig. 3B). In addition, autoradiographic binding studies on brain slices revealed binding sites for ^{35}S-labelled VP 4–9 that were anatomically distinct from the classical VP/OT receptors (Brinton et al., 1985a; De Kloet et al., 1985; Jurzak et al., 1993). This binding could not be competed for with VP or AVT (Jurzak et al., 1995). [^{35}S]VP 4–9 binding sites were mainly associated with circumventricular organs (organus vasculosum lamina terminalis, subfornical organ, nucleus tractus solitarii, arcuate nucleus, superior and inferior coliculi, tenth lobule of the cerebellum, choroid plexus). The pineal gland also displayed intense labeling as well as the pontine nuclei, pontine reticular nuclei, lateral mammillary nucleus and different nuclei in the brainstem. Localization of the metabolite receptors in brain areas lacking the blood-brain barrier or endowed with a leaky blood–brain barrier may explain the central effects induced by a peripheral

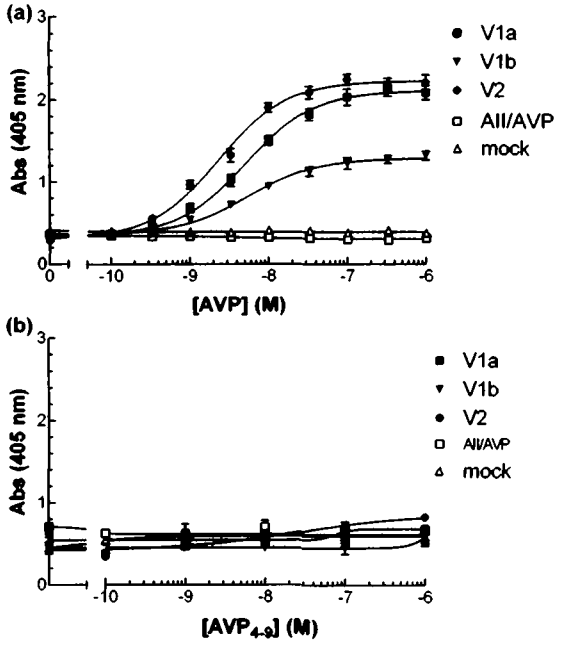

Fig. 3. Dose-response curves of VP receptor activation. A reporter gene with the galactosidase gene under control of a cAMP response element was transfected alone or cotransfected with the V_{1a}, V_{1b}, V_2 receptor or dual AII/VP receptor. Beta galactosidase activity was measured after 8-hour incubation with the indicated peptides using an ONPG assay (MacGregor et al., 1989) followed by absorbance measurement at 405 nm. (a) Shows activation of the V_{1a}, V_{1b} and V_2 receptors, but not the dual AII/VP receptor, by VP. In (b) it is demonstrated that none of the receptors is activated by VP 4–9. VP 4–8 had no effect on these receptors either (data not shown).

administration of these metabolites. Labeled VP 4–9 was also found to bind the kidney cortex in one study and also here VP was not able to block the binding of VP 4–9 (Brinton et al., 1985b). Many of the areas that bind [^{35}S]VP 4–9 in the brain and in the kidney could also be identified with an anti-idiotypic VP-antibody (Jurzak et al., 1992, 1993). Since the idiotypic VP-antibody used to generate the anti-idiotypic antibody specifically interacts with the C-terminal moiety of VP, the anti-idiotypes might preferentially recognize central VP 4–9-specific binding sites.

More recently Du et al. detected VP 4–8 binding

sites in hippocampal pyramidal cells and dentate granule cells using ^{35}S-labelled VP 4–8 in an autoradiographic study (Du et al., 1994a). Radioligand binding was also studied using labeled VP 4–8 on brain synaptosomal membranes (Du et al., 1994b). Binding was observed in all investigated brain regions but was most prominent in the amygdala, hypothalamus/hypophysis, and anterior cortex. VP was not able to displace the ^{35}S-labelled tracer. These observations indicate that VP 4–8 and VP 4–9 may bind to different receptors.

Another line of evidence that supports the existence of a distinct metabolite receptor comes from activation studies. VP 4–8/4–9 can induce increases in intracellular Ca^{2+} in about 8% of the neurons and astrocytes cultured from the subfornical organ or the organum vasculosum of the lamina terminalis (Jurzak et al., 1995). Only a sub-population of these metabolite sensitive cells is also responsive to VP indicating that VP and its metabolites activate different receptors. Indirect evidence suggests that the VP metabolite receptor may belong to the superfamily of G protein-coupled receptors. VP 4–8/4–9 stimulates IP turnover in hippocampal slices and primary cultures (Gu and Du, 1991; Diaz Brinton et al., 1994) and could stimulate accumulation of cAMP in hippocampal cultures (Diaz Brinton and Brownson, 1993).

Expression cloning identified recently two novel putative VP receptors. The first, VACM-1 (for vasopressin-activated calcium mobilizing receptor) is a 780 amino acid protein that confers vasopressin sensitivity on transfected COS-1 cells (Burnatowska-Hledin et al., 1995). Sequence analysis indicates that the VACM-1 protein lacks a signal peptide as well as transmembrane domains. VACM-1 is a member of the *cullin* gene family, an evolutionary old family of cell cycle regulators already present in yeast and *C. elegans*. Likely VACM-1 is as its *C. elegans* family members a cytoplasmic protein (Kipreos et al., 1996), which questions its role as a function VP receptor. The second putative receptor, AII/AVP (for dual angiotensin II and vasopressin receptor), confers sensitivity to both VP and angiotensin II when transfected in COS-1 cells (Ruiz-Opazo et al., 1995). This 481 amino acid protein was isolated by a homology screening strategy based on the molecular recognition theory (Bost et al., 1985; Mulcahey et al., 1986).

Whole cell binding analysis with [^{3}H]VP or [^{125}I]AII displayed saturable binding for both peptides with affinities in the low nM range. Functional studies showed that the putative AII/AVP receptor stimulates cAMP accumulation and that it has no effect on Ca^{2+} mobilization. However, receptor activation studied with a reporter gene assay sensitive to rises in cAMP and Ca^{2+} did not indicate any VP mediated increase in cAMP levels when the AII/AVP cDNA was transfected in HEK293 cells (Schoots, unpublished). The V_{1a}, V_{1b} and V_2 receptor showed clear activation by VP in this assay (Fig. 3A). VP 4–9 was not able to stimulate any of the receptors tested (Fig. 3B). Homology screening of protein databases using AII/AVP indicates some sequence homology with RNase inhibitors. Possibly, VACM-1 and AII/AVP do not encode vasopressin receptors themselves, but confer VP sensitivity via indirect and as yet unknown mechanisms.

It appears conceivable that receptors for vasopressin metabolites exist as distinct entities and that metabolism of VP renders a peptide that is inactive at the classical vasopressin receptors but active at the VP metabolite receptor(s). Clearly, it would be of great importance to isolate the cDNA encoding the putative vasopressin metabolite receptor(s). Expression cloning with cDNA made from tissue that is metabolite responsive and using the metabolite-induced increase in intracellular Ca^{2+} as a read-out may be the most direct approach to achieve this goal.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
Progress in Brain Research, Vol. 119

CHAPTER 2.2.3

Regulation of the synthesis and secretion of vasopressin

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We have developed a transgenic system that, for the first time, facilitates the monitoring of the regulatory dynamics of a central peptidergic system from transcription of a neuropeptide gene to the storage and release of the mature secretory product. Here we describe novel studies on the regulation of this system by physiological stimuli. The rat hypothalamic vasopressin (VP) mRNA responds in two ways to the functional demand imposed by an osmotic challenge. Firstly, the abundance of the VP RNA increases, and secondly, the size of the VP transcript increases as a consequence of a lengthening of the poly(A) tail. We have previously shown that chronic ingestion of 6-*n*-propyl-2-thiouracil (PTU), while not affecting plasma osmolality or VP mRNA size, results in a significant increase in the abundance of the hypothalamic VP mRNA. We now show that chronic PTU ingestion results in a dramatic increase in the abundance of the mRNA encoded by a modified rat vasopressin transgene that is expressed in rat vasopressinergic magnocellular neurons. This is accompanied by a significant depletion in neural lobe stores of a VP. However, this increase in transgene expression is accompanied by an increase in the proportion of transgene encoded products reaching the neural lobe - the pituitary content of a unique peptide encoded by the modified transgene does not change. These observations are further evidence in support of models of neurohypophyseal homeostasis that suggest that pituitary VP peptide levels passively reflect changes in hormone release and synthesis and that the availability of mRNA is the primary determinant of pituitary VP content in the basal state.

Vasopressin

Vasopressin (VP) is a neuropeptide hormone synthesised as a precursor prepropeptide, principally in magnocellular neurons whose cell bodies are located in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the mammalian hypothalamus (Swanson and Sawchenko, 1983). The VP precursor is packaged into secretory granules and is subject to cleavage and other modifications as it is transported from the cell bodies to storage in posterior pituitary nerve terminals (Brownstein et al., 1980). The mature peptide products are the nonapeptide VP, a putative carrier molecule termed neurophysin (NP) and a 39 amino acid glycopeptide (copeptin; CPP). These are stored in equimolar amounts in posterior pituitary terminals until neural inputs governed by physiological stimuli elicit their release (Renaud and Bourque, 1991). VP is a central component of the systems that regulate osmotic balance (Reeves and Andreoli, 1992). The physiological challenge of dehydration results in a rise in plasma osmolality that is detected by an undefined osmoreceptor mechanism. Subsequent excitation of the magnocellular hypothalamic neurons leads to a release of VP into the general circulation from posterior pituitary stores. VP travels through the blood

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stream to its targets that exhibit specific receptors. Particularly, through an interaction with V2-type receptors located in the kidney, VP increases the permeability of the collecting ducts to water. Thus, the hypothalamus (through VP) promotes water conservation by decreasing the amount of water lost in the urine. The related nonapeptide hormone oxytocin (OT) is also expressed in magnocellular neurons of the SON and PVN, but VP and OT are rarely found in the same cell (Mohr et al., 1988).

Physiological regulation of vasopressin gene expression

Studies on the physiological regulation of the VP gene expression have benefited from the exploitation of well-established paradigms for the modulation of the activity of vasopressinergic neurons. However, little is known about the mechanisms that regulate VP gene expression, in terms of either the synaptic regulation physiological status, or the downstream second messenger and transcription factor systems involved. VP gene expression in magnocellular cells is subject a hierarchy of controls. Osmotic stimuli such as dehydration (fluid deprivation) or salt-loading (the normal tap-water diet is replaced with a solution of 2% w/v NaCl), result in an increase in transcription (Murphy and Carter, 1990; Herman et al., 1991), a concomitant increase in VP mRNA abundance (Burbach et al., 1984; Zingg et al., 1986; Lightman and Young, 1987, Sherman et al., 1988) and an increase in the length of the VP RNA poly(A) tail (Carrazana et al., 1988; Carter and Murphy, 1989, 1991). The OT gene is similarly regulated by osmotic stimuli (Lightman and Young, 1987; Carter and Murphy, 1989; Van Tol et al., 1987) despite being expressed in distinct magnocellular neurons (Mohr et al., 1988). We have shown that the increases in VP mRNA abundance and poly(A) tail length can occur independently of each other (Carter and Murphy, 1989, 1991, 1993) and are probably separately regulated. For example, chronic ingestion of PTU, whilst not affecting plasma osmolality or VP mRNA size, results in a significant increase in the abundance of the hypothalamic VP mRNA (Carter et al., 1993).

Transgenic rodents and vasopressin

The hypothalamic VP system presents a classic example of the plasticity of gene expression in mature peptidergic neurons, within which some novel processes are operational. Whilst the molecular mechanisms underlying both the cell-specific expression and the physiological regulation of the VP gene are the subject of much current interest, these studies are hampered by the lack of convenient cell lines that express VP or OT appropriately. We have taken a transgenic approach, since this offers a bridge between molecular and functional analyses of responses that can only be studied in an intact organism (Murphy and Carter, 1992). We have studied transgenic rats, rather than mice, since it is the former that is the species of choice for studies in neuroscience and physiology. The anatomy of the rat brain is well mapped, and the structure, function and regulation of the rat HNS have been the subject of detailed study for many years. The large size of the rat makes it easily accessible for a whole range of physiological measurement and intervention, but its reproductive capacity and gestation time are equivalent to the mouse. This laboratory therefore adapted the standard fertilised one-cell mouse egg microinjection techniques to the rat, and generated transgenic rats bearing a derivative of the rat VP gene called 5-VCAT-3 that shows appropriate cell-specific and physiological expression in hypothalamic vasopressinergic magnocellular neurons (Zeng et al., 1994). 5-VCAT-3 consists of the rat VP structural gene, containing reporter sequences in exon III derived from the bacterial chloramphenicol acetyl transferase (CAT) gene, flanked by 5 kbp of upstream and 3 kbp of downstream sequences. The CAT sequences provide a unique nucleic acid reporter of transgene RNA expression, which we have used in situ hybridisation and Northern analyses (Zeng et al., 1994). Further, translation of the reporter places a unique hexadecapeptide (DRSAGYYGLFKDRKEK, abbreviated to DR-12-EK) at the C-terminus of a modified prepropeptide (Fig. 1). Exons I and II of the transgene are normal, and thus the signal peptide, the VP nonapeptide and the regions of NP encoded by these exons are the same as in the wild-type prepropeptide. 72 bp of exon III are

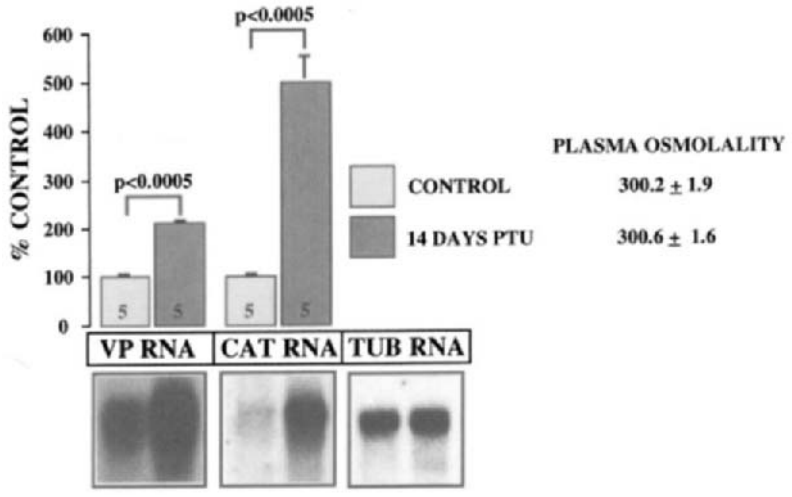

Fig. 1. Levels of VP and transgene RNAs in hypothalami of control and PTU treated 5-VCAT-3 transgenic rats. Comparison of hypothalamic levels of transcripts encoded by the 5-VCAT-3 transgene (CAT), the VP gene and the α-tubulin gene (TUB) in hypothalami of control and 14 day PTU treated transgenic rats. Quantitation of VP and 5-VCAT-3 (VP-CAT) RNAs in hypothalami of control transgenic rats, and transgenic rats subject to 14 days of PTU ingestion. RNA levels were determined by Northern analysis and corrected against α-tubulin RNA. VP and CAT RNA levels are expressed as a percentage of the levels in control transgenic animals. Group numbers are shown in each column (1 animal per group). Representative Northern blots are shown below the graphs. Each lane represents 25 μg of total cellular hypothalamic RNA. Methods: All animals were bred in Singapore. We have described two lines of Sprague–Dawley rat bearing the 5-VCAT-3 transgene (lines 1 and 2). Line 1, which bears 40 copies of the transgene per haploid genome (Zeng et al., 1994), was used in the studies described here. Animals were cared for in accord with NIH guidelines. Southern blot positive obligate heterozygous animals were used in all experiments. It has recently been shown that rats experience a dramatic increase in pituitary VP content between 3 and 20 weeks of age, after which levels stabilise (Fitzsimmons et al., 1994). We therefore used 30 week old animals in the experiments described here. Age-matched wild-type Sprague–Dawley rats were compared with transgenic animals. Hypothyroidism was induced by treatment with 6-*n*-propyl -2-thiouracil (PTU), which was administered in drinking water (0.05% (w/v) in tap water for 14 days; Ladenson et al., 1986). Control animals continued to receive tap water. Following treatment, rats were killed by decapitation, and trunk blood was collected into heparinised tubes. The hypothalamus and the neurointermediate lobe of the pituitary (NIL) were rapidly removed. The hypothalamus was snap-frozen on dry ice and stored at −80°C prior to the extraction of RNA. The NIL was placed in 0.5 ml of 0.1 M HCl and processed immediately for peptide assays. Heparinised trunk blood was centrifuged and plasma osmolality and was assayed (Wescor Vapor Pressure Osmometer, Logan, UT). RNA was extracted from frozen hypothalami and analysed by Northern blotting (Murphy et al., 1989). The specific CAT and VP probes used in this study have been described (Zeng et al., 1994). Linear range autoradiograms were scanned by laser densitometry (LKB Ultroscan). Levels of VP and transgene RNA were calculated relative to the level of the α-tubulin RNA control, and expressed as a percentage of the mean of the tap water control groups (mean ± SEM). Significance was determined using Student's *t*-test.

deleted from the transgene as compared to the normal VP gene, and these are replaced by sequences from the CAT gene (Zeng et al., 1994). The C-terminal portion of NP encoded by transgene exon III is intact (thus the whole of NP is wild-type), as is the cleavage signal that separates NP from CPP. The first 13 residues of CPP are intact (including the unique glycosylation site) but the last 26 amino acids of CPP are replaced by a novel 16 amino acid peptide (DR-12-EK). Although most of the prepropeptide is left intact, this substitutes a relatively hydrophilic peptide for the more hydrophobic region found in the wild-type CPP molecule.

We have raised an antibody against the DR-12-EK 'tag' and, using immunohistochemistry, electron microscopy, RIA and HPLC, have shown that the transgene RNA is translated into a protein product found, in a processed form, in secretory

granules in the posterior pituitaries of transgenic rats (Waller et al., 1996). Disruption of the C-terminus of the VP precursor by the peptide 'tag' is thus well tolerated, and does not disrupt VP production or disturb salt and water balance.

The physiological regulation of the 5-VCAT-3 transgene has been well defined (Zeng et al., 1994; Waller et al., 1996). An osmotic stimulus that increases the level of the endogenous VP RNA 2-fold increase the level of the transgene RNA 10–20-fold. However, whereas the length of the poly(A) tail of the endogenous VP RNA increases following an osmotic stimulus (see above) the size of the transgene RNA is unchanged, because of its altered structure. An osmotic stimulus increases hypothalamic DR-12-EK levels, but changes in posterior pituitary DR-12-EK levels were more complex. After 5 days salt-loading DR-12-EK levels fell, as would be expected if its release was co-ordinate with that of VP. However, after 10 days of salt-loading, posterior pituitary DR-12-EK levels increased, despite the lower level of VP. This probably reflects the greater response of the transgene to osmotic challenge at the RNA level, increasing the proportion of DR-12-EK-containing translation products transported to the posterior pituitary relative to those derived from the endogenous gene.

PTU regulation of 5-VCAT-3 expression

We have now asked if another stimulus of VP gene activity, chronic ingestion of PTU, also affects the expression of the 5-VCAT-3 transgene and its peptide products. We have compared the effect of PTU ingestion on the hypothalamic content of the endogenous VP RNA and the transgene encoded mRNA in 5-VCAT-3 rats (Fig. 1). As previously reported (Carter et al., 1993), the VP RNA increases two fold in abundance following PTU ingestion, but there is no change in the size of the transcript. The abundance of the transgene encoded transcript increases 5-fold.

We chose to study hypothyroidism induced by PTU ingestion, since we previously showed this treatment leads to a significant 2-fold increase in the abundance of the VP RNA in the hypothalamus (Carter et al., 1993). This was confirmed in the present study. As with wild-type rats (Carter et al., 1993), PTU ingestion by 5-VCAT3 transgenic rats is not accompanied by an increase in plasma osmolality (Table 1). Whilst 14 days of PTU ingestion results in significant reductions in NIL VP and OT content, the level of DR-12-EK did not change significantly (Table 1).

The mechanism of PTU induced VP depletion merits some comment. We have previously shown that the increase in VP gene expression caused by PTU ingestion is not related to the induction of hypothyroidism (Carter et al., 1993) - neither surgical thyroidectomy nor PTU injection affected VP RNA levels in the hypothalamus (Carter et al., 1993). Rather, we suggested that VP gene upregulation may be functionally correlated with impaired drinking of the unpalatable PTU solution, even though this did not affect plasma osmolality. We have confirmed these observations and, further, shown that PTU ingestion cause a depletion of VP (and, to a lesser extent, OT) stores in the posterior pituitary. Although the impaired drinking in this paradigm is not a dehydration stimulus, in the sense that plasma osmolality is not increased, it is likely that the VP release observed is responsible for maintaining normal levels of hydration.

The increase in 5-VCAT-3 expression as a consequence of PTU ingestion was more marked than for the endogenous wild type VP gene. This is similar to the exaggerated response of the 5-VCAT-3 transgene to dehydration or salt-loading (Zeng et al., 1994; Waller et al., 1996). This exaggerated response of the transgene RNA level to physiological stimuli may be a transcriptional or a post-transcriptional phenomenon. We have previously postulated that the exaggerated response of the 5-VCAT-3 to osmotic challenge may be due to the release of the transgene from the action of one or more repressor sequences, absent from the transgene but present in the normal context of the VP gene, which attenuate the response of the VP gene to physiological stimuli. However, it is also possible that the insertion of CAT sequences into exon III of the modified transgene RNA has altered its response to the normal post-transcriptional regulatory processes. We have previously suggested (Waller et al., 1996) that these effects may also be related to differences in the poly(A) tail, which, for

Table 1
Physiological responses of 5-VCAT-3 transgenic rats to PTU ingestion[a]

	Control	PTU ingestion (14 days)	
Plasma osmolality (mosm/kg)	300.2 ± 1.9 (5)	300.6 ± 1.6 (5)	
NIL VP (ng/organ)	897 ± 87 (5)	281 ± 51 (5)	$P < 0.025$
NIL OT (ng/organ)	1413 ± 251 (5)	717 ± 160 (5)	$P < 0.0005$
NIL DR-12-EK	37.4 ± 6.5 (5)	51.1 ± 9.4 (5)	

[a] Methods. Animals were prepared as described in the legend to Fig. 1. The preparation of the rabbit anti-DR-12-EK sera has been described (Waller et al., 1996). Briefly, rabbits were inoculated with the following peptide: CDRSAGYYGLFKDRKEK (C-DR-12-EK). This corresponds to the predicted C-terminal sequence of the modified CPP encoded by 5-VCAT-3 (DR-12-EK) with an additional N-terminal Cys residue to facilitate coupling to KLH. Antibodies were raised in rabbits using an IACUC approved protocol. Following a primary injection of 150 μg of antigen with Freund's Complete Adjuvant, each cycle consisted of 5 boosts of 100 μg of antigen with Freund's Incomplete Adjuvant and 4 bleeds (Animal Pharm Services Inc., Healdsburg, Ca). Unpurified sera from rabbit 21, bleed 26 (Ab21/26) was used to develop an RIA for DR-12-EK as described (Waller et al., 1996). The C-DR-12-EK peptide was iodinated using Iodogen and purified by gel filtration and/or reverse-phase HPLC (Tannahill et al., 1988). Samples (100 μl) of peptide standard (0.01–25 ng) or tissue extracts were diluted in RIA buffer (0.1 M Tris–HCl, pH 7.4, containing 3 mg/ml BSA) in duplicate, and mixed with 100 μl radioiodinated DR-12-EK and antiserum Ab21/26 (100 μl) to a final dilution of 1:5000. After incubation at 4°C overnight, bound tracer was precipitated by addition of polyelthylene glycol as previously described (Fairhall and Robinson, 1989). Under these conditions, specific binding was >40%, non-specific binding was <5% and the assay sensitivity was 20 pg/tube, expressed in terms of the synthetic peptide standard. Extracts were assayed over four doubling dilutions in duplicate and shown to dilute in parallel to the standard curve. The same extracts were also assayed for VP and OT content using specific RIAs (Horn et al., 1985; Robinson, 1980). Data are expressed as ng of peptide per organ (±SEM). The number of groups is shown in parentheses. Significance was determined using Student's *t*-test.

the endogenous RNA but not the transgene RNA (Zeng et al., 1994), increases in length upon osmotic stimulation. This could endow the wild-type transcripts with a longer half-life than their transgene RNA counterparts. The present results argue against this hypothesis, since PTU ingestion had no effect on the poly(A) tail length of either the endogenous or the transgene RNA. It is unlikely, therefore, that the exaggerated effect of physiological stimuli on transgene RNA level is simply related to polyadenylation.

The substitution of the DR-12-EK peptide 'tag' in the VP precursor also makes it possible to measure the pituitary content of the transgene translation products, and compare to the levels of VP which may derive from both VP and 5-VCAT-3 genes. Although VP RNA levels were markedly increased, the pituitary content of VP (and OT) fell substantially following 14 days of PTU ingestion, consistent with prolonged release of these peptides. However, the level of DR-12-EK in the posterior lobe of the pituitary actually increased. A similar effect was previously observed in response to a prolonged osmotic stimulus. Pituitary DR-12-EK levels fell initially, coordinate with the release of VP. However, DR-12-EK levels increased when the osmotic stimulus was maintained for 10 days, despite continued low VP or OT levels (Waller et al., 1996). We suggested that this was a consequence of the much greater increase in transgene RNA levels over endogenous VP RNA levels, occasioned by this physiological stimulus, thus increasing the proportion of DR-12-EK-containing translation products transported to the posterior pituitary relative to those derived from the endogenous gene. This idea has been strengthened by the observations for the present study, which show that this increase in transgene expression is also reflected in a relative increase in the proportion of products coded by transgene transcripts, reaching the neural lobe. These observations are further evidence in support of models of neurohypophyseal homeostasis that suggest that pituitary VP peptide levels passively reflect changes in hormone release

and synthesis, and that the availability of mRNA is the primary determinant of pituitary VP content in the basal state (Fitzsimmons et al., 1992, 1994).

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SECTION 3

Cellular actions of vasopressin

Molecular actions and responses to vasopressin
Electrophysiological actions of vasopressin

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
Progress in Brain Research, Vol. 119

CHAPTER 3.1.1

Signal transduction pathways of the human V_1-vascular, V_2-renal, V_3-pituitary vasopressin and oxytocin receptors

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Vasopressin (VP) and oxytocin (OT) are cyclic nonapeptides whose actions are mediated by stimulation of specific G protein-coupled receptors (GPCRs) currently classified into V_1-vascular (V_1R), V_2-renal (V_2R) and V_3-pituitary (V_3R) VP receptors and OT receptors (OTR). The recent cloning of the different members of the VP/OT family of receptors now allows the extensive characterization of the molecular determinants involved in ligand binding and signal transduction pathways coupled to a given VP/OT receptor subtype in stably transfected mammalian cell lines.In this article, we review the present knowledge of the signal transduction pathways coupled to the different VP/OT receptor subtypes and we present new observations derived from the study of each human VP or OT receptor subtype stably expressed in CHO cells.

Introduction

The neurohypophysial hormones vasopressin (VP) and oxytocin (OT) are cyclic nonapeptides whose actions are mediated by stimulation of specific G protein-coupled receptors (GPCRs) currently classified into V_1-vascular (V_1R), V_2-renal (V_2R) and V_3-pituitary (V_3R) VP receptors and OT receptors (OTR) (Michell et al., 1979; Jard, 1983; Thibonnier, 1992, 1993a,b). The cardiovascular and renal effects of VP are reasonably well characterized (Johnston, 1985; Goldsmith, 1987). Conversely, VP actions at the level of the central nervous system where it acts as a neurotransmitter or neuromodulator controlling major functions including blood pressure, memory, body temperature, brain development, and release of pituitary hormones remain to be documented further. Similarly, a more detailed understanding of the actions and signal transduction pathways of the OT receptor is required.

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Recently, several groups cloned various members of the family of human and animal VP/OT receptors (Birnbaumer et al., 1992; Kimura et al., 1992; Lolait et al., 1992, 1995; Morel et al., 1992; Gorbulev et al., 1993; De Keyser et al., 1994; Mahlmann et al., 1994; Sugimoto et al., 1994; Thibonnier et al., 1994; Hutchins et al., 1995). Stable expression of these cloned receptors in heterologous mammalian cells now allows the detailed characterization of the signal transduction pathways coupled to VP or OT stimulation of a given receptor subtype, without the possible interference from other receptor subtypes and endogenously bound hormone.

Functional characterization of GPCRs (β1- and β2-adrenergic receptors, luteinizing hormone receptor, the VP V_2-renal receptor) in mammalian cell lines indicates that a single receptor type can activate multiple second messenger pathways through interaction with one or more G proteins, which in turn appear to be modulated by the number of receptors being expressed (George et al., 1988; Zhu et al., 1994; Birnbaumer, 1995). Similarly, the

endogenous human TSH receptor not only activates G_s and $G_{q/11}$ but also members of the G_i and G_{12} families, indicating a complex modulation of downstream pathways (Laugwitz et al., 1996). Thus, GPCRs have the potential to couple to multiple signaling pathways, with the activation of a specific pathway being dependent on both the receptor density and the agonist concentration. These multiple pathways may play an important role in regulating the effectiveness of the signals in different tissues. Depending on the expression level of the receptor and the local concentration of the signal, the final outcome following agonist binding may differ.

In this chapter we review the known cellular signal transduction pathways linked to the activation of the human V_1-vascular, V_2-renal, V_3-pituitary VP, and OT receptors. In addition, we present new information on the ligand binding profile, mitogenic effect, and activation of the MAP kinase pathway by the human VP/OT receptor subtypes stably expressed in mammalian cells.

Precedents of the signal transduction pathways coupled to the human V_1R, V_2R, V_3R and OTR

V_1-vascular receptor

The V_1-vascular receptor expressed in the liver, vascular smooth muscle cells, and the testis is the product of the same gene, undergoing identical splicing (Thibonnier et al., 1995). This V_1-vascular receptor is also expressed in several tissues or organs including blood platelets, adrenal cortex, kidney, reproductive organs, spleen, adipocytes, the brain and various cell lines (3T3, A10, WRK-1 and A7r5). VP binding to the V_1-vascular receptor leads to the activation of phospholipases C, D, and A_2, the production of inositol 1,4,5-triphosphate and diacylglycerol, the activation of protein kinase C, the mobilization of intracellular calcium, the influx of extracellular calcium via receptor-operated Ca^{2+} channels, and the activation of the Na^+-H^+ exchanger (Thibonnier, 1992; Briley et al., 1994). No stimulation of cAMP accumulation is noted after stimulation of the V_1-vascular receptor. The secondary nuclear signal mechanisms triggered by activation of V_1-vascular receptors include induction of immediate-early response genes expression and protein synthesis, leading to cellular hypertrophy and increased cell protein content (Geisterfer and Owens, 1989). Indeed, activation of V_1-vascular receptors leads to a mitogenic response in vascular smooth muscle cells, 3T3 cells, renal mesangial cells, hepatocytes and adrenal glomerulosa cells. These responses are specifically blocked by V_1-vascular receptor antagonists of peptide and non-peptide nature. The G proteins coupled to the V_1-vascular receptor are mainly members of the $G_{q/11}$ family, but also of the G_i family, as some of the signals activated by V_1-vascular receptors stimulation (e.g. phospholipase A_2 activation) are reduced by pertussis toxin pretreatment (Thibonnier et al., 1995). Shortly after agonist binding, VP receptor internalization occurs and may contribute to receptor desensitization (Fishman et al., 1985; Lutz et al., 1993). Recently, it was reported in rat vascular smooth muscle cells and 3Y1 fibroblasts that the stimulation of V_1-vascular receptors lead to the activation of the MAP kinase pathway (Granot et al., 1993; Nishioka et al., 1995). The activation of MAP kinase is pertussis toxin-insensitive, via PKC-dependent and -independent pathways. The latter seems to involve the phosphatidylinositol 3-kinase (Nishioka et al., 1995). Phosphorylation and dephosphorylation of the V_1-vascular receptor and their role in the desensitization/resensitization processes remains to be demonstrated.

V_2-renal receptor

The V_2-renal receptor is expressed in the medullary portion of the kidney (as well as in the MDCK and LLC-PK_1 cell lines) where it mediates the antidiuretic effect of vasopressin. VP binding to the V_2-renal receptor leads to the sequential coupling of the cholera toxin-sensitive G protein G_s, activation of adenylyl cyclase, production of cAMP and activation of protein kinase A, promoting the insertion of water channels (aquaporins) into the luminal surface of the renal collecting tubule cells (Hayashi et al., 1994; Bichet et al., 1995). An elegant work by Kojro and Fahrenholz, 1995) suggests that enzymatic cleavage of the ligand-occupied V_2-renal receptor by a metalloprotease leads to a

major alteration of the binding site contributing to the termination of signal transmission.

V_3-pituitary receptor

This VP receptor subtype was designated as V_3 or V_{1b} (Baertschi and Friedli, 1985; Jard et al., 1986).The V_3-pituitary receptor was described initially in corticotroph cells where it potentiates the release of ACTH; however, recent RT-PCR experiments suggest its presence in other tissues such as the kidney, the pancreas and the adrenal medulla (De Keyser et al., 1994, 1996; Lolait et al., 1995; Grazzini et al., 1996). Prior to the cloning and functional expression of the human pituitary V_3R, studies of the binding characteristics and signal transduction pathways activated following binding of VP to this receptor have been hampered by its limited availability. Initial observations were made using either animal (rat, pig, sheep) freshly isolated cells (Giguere and Labrie, 1982; Antoni, 1984; Knepel et al., 1984; Baertschi and Friedli, 1985; Aguilera, 1994) or samples of human corticotroph adenomas (Levy et al., 1990). In these studies, occupancy of V_3Rs by agonists triggered the sequential activation of phospholipase C and protein kinase C, the mobilization of intracellular free calcium, the phosphorylation of the myristoylated alanine-rich C kinase substrate and secretion of ACTH (Levy et al., 1990; Liu, 1994; Liu et al., 1994). Conflicting data regarding coupling of the V_3R to adenylyl cyclase have been reported (Giguere and Labrie, 1982; Holmes et al., 1984; Knepel et al., 1984). No information was available regarding the nature of the G-protein(s) and the kinases-phosphatases coupled to the V_3R, as well as the eventual mitogenic role of this receptor. Studies of ligand binding profile, coupling to phospholipase C and adenylyl cyclase revealed a unique pharmacological profile for this pituitary receptor, distinct from those of the V_1R and the V_2R subtypes. A recent pharmacological characterization of the porcine pituitary VP receptor with cyclic and linear peptide VP antagonists confirmed that the pituitary and liver VP binding sites were dissimilar, both cyclic and linear V_1 antagonists having in general a much lower affinity for the pituitary receptor than for the liver one (Arsenijevic et al., 1994). We have recently completed a comprehensive characterization of the signal transduction pathways linked to the human V_3-pituitary receptor expressed in CHO cells (Thibonnier et al., 1997). Depending on the level of expression of the receptor, the V_3-pituitary receptor couples to members of the $G_{q/11}$ family, alone or in combination with G_i, and may also recruit G_s. Thus, the human V_3R has a pharmacological profile clearly distinct from that of the human V_1R and V_2R and activates several signaling pathways via different G proteins, depending on the level of receptor expression. The increased synthesis of DNA and cAMP levels observed in cells expressing medium and high levels of V_3Rs, respectively, may represent important events in the tumorigenesis of corticotroph cells (Thibonnier et al., 1997).

OT receptor

The OT receptor is expressed in the uterus, the mammary gland, the ovary, the brain, the kidney and lactotroph cells. OT binding to its receptor leads to phospholipase C activation, calcium mobilization and stimulation of phosphatidyl inositol turnover (Kimura and Saji, 1995). A recent publication by Ohmichi et al. (1995) indicates that stimulation of the OT receptor of human uterine myometrial cells induces MAP kinase phosphorylation through a pertussis toxin-sensitive G protein. In human myometrial cells, the OT receptor activates phospholipase Cβ by interacting with at least two types of G proteins, a member of the pertussis toxin-sensitive G_i family and a member of the pertussis toxin-insensitive $G_{q/11}$ family (Phaneuf et al., 1996).

Radioligand binding characteristics of the human V_1R, V_2R, V_3R and OTR

Reagents

Standard reagents, unless stated otherwise, were from Sigma (St. Louis, MO). CHO cells were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media and geneticin were from Gibco-BRL (Grand Island, NY). Fetal bovine serum was from Hyclone (Logan,

UT). Iodogen (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril) was from Pierce Chemical (Rockford, IL). Restriction and modification enzymes were from Boehringer Mannheim (Indianapolis, IN) or NE Biolabs (Beverly, MA). [^{125}I]Na (activity = 131 mCi/ml), [^{3}H]VP (specific activity = 48 Ci/mmol), [^{3}H]OT (specific activity = 35 Ci/mmol), and [^{3}H]thymidine (specific activity = 1 mCi/ml) were obtained from NEN Dupont (Wilmington, DE). The pBluescript II phagemid KS, and XL2-Blue E. coli strain were from Stratagene (La Jolla, CA). The expression vectors pZeoSV and pcDNA3.1 and the antibiotic zeocin were from Invitrogen (San Diego, CA). Arginine vasopressin (VP), oxytocin (OT), arginine vasotocin (AVT), lysine vasopressin (LVP), and most of the peptide V_1, V_2, V_1/V_3, and OT agonists and antagonists were from Bachem (Torrance, CA) unless indicated otherwise. The non peptide V_1 antagonist SR 49059 (batch number MY10-075) was provided by Dr. C. Serradeil-Le Gal, Sanofi Recherche (Toulouse, France). The non-peptide rat V_1 antagonist OPC 21268 (batch number 93F92M), and the non-peptide V_2 antagonist OPC 31260 (batch number 93D96M) were provided by Dr. J.F. Liard (Otsuka America Pharmaceutical, Rockville, MD). The linear V_1 antagonists 4-hydroxy-phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (OHPhaaGln) and tert-butylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Arg-NH_2 (tBaa), the V_2 antagonist d$(CH_2)_5$[D-Ile^2-Ile^4-Ala-NH_2]VP, and the linear V_1/V_3 antagonist phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2 were provided by Dr. Maurice Manning from the Medical College of Ohio in Toledo. The linear V_1 VP antagonist phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Tyr-NH_2, custom-synthesized by Bachem (Torrance, CA), was radioiodinated ([^{125}I]TyrPhaa) with the iodogen technique and purified by HPLC as previously described (Thibonnier et al., 1993).

cDNA constructs

The human V_1R clone was isolated by screening a human liver cDNA library as described before (Thibonnier et al., 1994). The human V_2R clone was generated by PCR from human kidney cDNA (Clontech human kidney QUICK-Clone cDNA, #7112-1, lot #48570) using the sense primer 5′-CATCATGGGCCCACCATGCTCATGGCG-3′ (which introduces an ApaI restriction site upstream of the start codon and includes the first 12 nucleotides in the open reading frame) and the antisense primer 5′-ACACCCAGCTCAGTGAG-CTG-3′ (downstream from the stop codon and a native ApaI site, including nucleotides 1347–1366 in Birnbaumer et al., 1992). The oligonucleotides were prepared in a synthesizer (Applied Biosystems, Foster City, CA) and purified over OPC cartridges following the manufacturer recommendations. The PCR reaction (final volume 100 µl) contained 20 pmol of each primer, 2.5 units of Taq polymerase, 5 ng of human kidney cDNA, 10 µM of each dNTPs in buffer 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin (pH 7.4). After initial denaturation at 95°C for 5 min, 30 cycles were run (95°C 1 min, 56°C 1 min, 72°C 1.5 min) with a final extension at 72°C for 15 min. The V_3R cDNA clone we isolated previously (De Keyser et al., 1994) was derived from a human pituitary tumor. To rule out the possibility of mutations within the receptor sequence because of its tumoral origin, we generated by PCR the human V_3R cDNA from normal human pituitary gland cDNA (human pituitary gland QUICK-clone cDNA from Clontech, Catalog #7173-1, lot #46910). The sense primer was 5′-TGCTTG-AAGTCCTCTGAACG-3′ (nucleotides −167 to −148 in Sugimoto et al., 1994) and reverse primer was 5′-AAGACAGCA CCATCCTAGGC-3′ (nucleotides 1578 to 1597 in (Sugimoto et al., 1994), downstream from the stop codon and a native SpeI site). The PCR reaction with 5 ng of normal human pituitary gland cDNA used the same conditions as those described above for the human V_2R. The PCR product was purified with the GeneClean II (Bio 101, Vista, CA). An SpeI restriction site was introduced 140 bp upstream of the initiation codon by PCR and the SpeI-SpeI cDNA fragment was digested, purified and sequenced. The human OTR clone was generated by PCR from human ovary cDNA (Clontech human ovary QUICK-Clone cDNA, #7122-1, lot #48870) using two sets of primers. The first set of primers was used to amplify the 5′ region of the OTR: sense primer 5′-TCAACTTTAGGTTCGCCTGC-

3′ (nucleotides −271 to −252 in Kimura et al., 1992) and reverse primer 5′-TCTTGAAGCTGA-TAAGGCCG-3′ (nucleotides 659–678 in Kimura et al., 1992). The second set of primers was used to amplify the 3′ region of the OTR: sense primer 5′-CGGCCTTATCAGCTTCAAGA-3′ (nucleotides 659–678 in Kimura et al., 1992) and reverse primer 5′-TCATCTTCCATCATGGAGGC-3′ (nucleotides 1321–1340 in Kimura et al., 1992). The PCR reaction conditions were identical to those above except for inclusion of 4% DMSO for amplification of the GC-rich 5′ region of the OTR. After ligation and digestion, the BamHI-KpnI fragment of the OTR was purified and sequenced. Nucleotide and amino acid sequences were analyzed with the computer package GeneWorks on a Macintosh computer (IntelliGenetics, Mountain View, CA).

cDNAs subcloning and sequencing

The EcoRI-EcoRI V_1R, ApaI-ApaI V_2R, SpeI-SpeI V_3R, and BamHI-KpnI OTR constructs were ligated into pBluescript II phagemid vectors prior to transformation of XL2-Blue *E. coli* strain. Double-stranded DNA sequencing was performed with the Taq Dye Deoxy Terminator cycle sequencing kit and a model 373A sequencer from Applied Biosystems (Foster City, CA).

Selection and stable expression of VP/OT receptors in CHO cells

Stable transfection of CHO cells with the pZeoSV or pcDNA3.1 expression vector containing the sequence of the human V_1R, V_2R, V_3R or OTR cDNA clones we isolated as described above was performed using the calcium phosphate precipitation method (Thibonnier et al., 1994). CHO-V_1, CHO-V_2, CHO-V_3 and CHO-OT cells were grown in medium F12 supplemented with 10% fetal calf serum, selected with the neomycin analogs geneticin or zeocin, and purified by the limiting dilution technique. Clones expressing various densities of VP/OT receptors were studied by radioligand saturation and competition binding experiments as well as measurement of thymidine uptake and MAP kinases activation as described below.

Radioligand binding assays

Control and transfected CHO cells were grown to confluence in 24-well dishes and washed twice with PBS + 10 mM $MgCl_2$ + 0.2% BSA (pH 7.4). Saturation binding experiments of VP/OT receptors of transfected CHO cells were performed in 24-well dishes in duplicate with increasing concentrations of [^{3}H]VP ± 1 μM unlabeled VP or of [^{3}H]OT ± 1 μM unlabeled OT (Thibonnier et al., 1994). Affinity (K_d) and capacity (B_{max}) of the VP/OT receptors were calculated by a nonlinear least square analysis program (Thibonnier and Roberts, 1985). Data were expressed as mean ± SEM. Protein concentration was measured with Pierce's BCA reagent using albumin as an internal standard. Competition binding experiments were performed as described before (Thibonnier et al., 1994) using one fixed concentration of [^{125}I]TyrPhaa for CHO-V1 cells, [^{3}H]VP for CHO-V2 cells and CHO-V3 cells or [^{3}H]OT for CHO-OT cells and increasing concentrations of unlabeled peptide and non-peptide VP/OT analogs (n = 3–5 for each analog) for 30 min at 30°C. IC_{50} values were derived from non linear least square analysis and K_i values were calculated by the equation of Cheng and Prusoff: $K_i = IC_{50}/(1 + L_f/K_d)$.

Stable expression of the human VP/OT receptors in CHO cells allowed us to carry out an extensive ligand binding characterization of these four human VP/OT receptor subtypes in the same mammalian cell line. CHO cells do not express endogenous VP/OT receptors and each clone tested in our experiments expressed a single VP/OT receptor subtype without any interference from the other subtypes as one may observe in homologous hosts.

The human V_1-vascular (CHO-V1), V_2-renal (CHO-V2), V_3-pituitary (CHO-V3) and oxytocin (CHO-OT) receptors expressed in the same mammalian CHO cell line have clearly distinct binding profiles (Table 1). As expected, VP is the endogenous ligand having the highest affinity for the human V_1-vascular, V_2-renal and V_3-pituitary receptors, whereas OT is the endogenous ligand with the highest affinity for the human OT receptor.

Review of the results for the CHO-V1 cell line indicates that all the peptide V_1 antagonists tested in our experiments display a high affinity for the V_1-

Table 1
Affinity (K_i in nM) of VP/OT structural analogs for the human V_1-vascular, V_2-renal, V_3-pituitary VP, and OT receptors stably expressed in CHO cells[a]

Compound	V_1-vascular	V_2-renal	V_3-pituitary	OT
Endogenous hormones				
VP	1.7	1.1	1.1	16
AVT	5.0	6.2	10	1.9
Oxytocin	64	167	1782	0.9
LVP	2.3	3.3	2.9	25
V_1 *antagonists*				
$d(CH_2)_5$Tyr(Me)VP	1.6	82	359	3.0
Phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Tyr-NH_2	2.4	1805	798	13
Phenylacetyl D-Tyr(Et)-Phe-Gln-Asn-Lys-Pro-Arg-NH_2	0.8	302	31	5.3
4-OH-phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2	0.5	428	2.2	0.4
Phenylacetyl-Dhyphen;Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2	0.8	282	9.4	1.14
tBaa-D-Tyr(Et)Phe-Val-ASn-Lys-Pro-Arg-NH_2	0.8	54	364	1.76
SR 49059	1.1	119	129	15
OPC21268	8800	32975	No displacement	185
V_2 *agonists*				
DDVP	21	2.7	22	89
DVDVP	10	0.8	25	290
V_2 *antagonists*				
$d(CH_2)_5$[D-Ile^2-Ile^4-Ala-NH_2]VP	53	88	541	67
$d(CH_2)_5$[D-Ile^2-Ile^4-Arg^8-Ala^9-NH_2]VP	30	76	650	33
OPC31260	142	12	14254	1561
V_1/V_3 *antagonists*				
Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2	0.6	259	16	49
Deamino^1(D-3-(Pyridyl)Ala^2-Arg^8)vasopressin	4.3	6422	50	192
Oxytocin antagonists				
$d(CH_2)_5$[O-Me-Tyr^2-Thr^4-Orn^8]vasotocin	7.6	5964	28700	0.6
$d(CH_2)_5$[O-Me-Tyr^2-Thr^4-Orn^{8}Tyr^9-NH_2]vasotocin	3.9	929	10229	0.5

[a] Affinity constants (K_i in nM) were obtained using the V_1-vascular ligand [^{125}I]phenylacetyl-D-Tyr(Et)-Phe-Val-Asn-Lys-Pro-Tyr-NH_2 for the V_1R, [^{3}H]VP for the V_2R and V_3R, and [^{3}H]OT for the OTR. Each value represents the mean of 3–8 independent experiments (data for V_1R, V_2R and V_3R are from our previous work (Thibonnier et al., 1997).

vascular receptor. Several compounds have a greater affinity than VP itself (K_i = 1.7 nM), the V_1 antagonist 4OH-Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 displaying the best affinity for the V_1R (K_i = 0.5 nM). The non-peptide compound OPC21268, which was characterized as a V_1 antagonist in the rat (Yamamura et al., 1991), displays a weak affinity for the human V_1-vascular

receptor as well as for the V_2-renal and V_3-pituitary receptors. The non-peptide V_1 antagonist SR49059 has an excellent affinity for the human V_1-vascular receptor (K_i = 1.1 nM). Interestingly enough, the two V_2-renal agonists (dDVP and dVDVP) have an affinity for the human V_1-vascular receptor which is not negligible (K_i = 21 and 10 nM, respectively). Similarly, the two peptide V_2-renal antagonists tested in our experiments ($d(CH_2)_5$[D-Ile^2-Ile^4-Ala-NH_2]VP and $d(CH_2)_5$[D-Ile^2-Ile^4-Arg^8-Ala^9-NH_2]VP) show a better affinity for the human V_1R (K_i = 53 and 30 nM) than for the human V_2R (K_i = 88 and 76 nM). The two available "V_3 antagonists" (phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2 and deamino[1](D-3-(pyridyl)Ala^2-Arg^8)-vasopressin) exhibit a significantly higher affinity for the V_1R than for the V_3R, thus confirming the current lack of potent and selective VP V_3 antagonists. The two peptide OT antagonists tested in our experiments ($d(CH_2)_5$[O-Me-Tyr^2-Thr^4-Orn^8]vasotocin and $d(CH_2)_5$[O-Me-Tyr^2-Thr^4-Orn^8-Tyr^9NH_2]vasotocin) also display a high affinity for the human V_1R (K_i = 7.6 and 3.9 nM).

Review of the results for the CHO-V2 cell line indicates that none of the V_1-vascular antagonists has a good affinity for the V_2R. The two peptide V_2-renal agonists dDVP and dVDVP have a good affinity for the human V_2R (K_i = 2.7 and 0.8 nM respectively), but they are not very selective as they also bind to the human V_1R and V_3R with dissociation constants in the 10–25 nM range. The non-peptide compound OPC31260, which was found to be a V_2 antagonist in the rat (Ohnishi et al., 1993), also displays an acceptable affinity and selectivity for the human V_2-renal receptor (K_i = 12 nM), whereas the two non-peptide V_1 antagonist SR49059 and OPC21268 have a poor affinity for the human V_2R. Both the V_3 and the OT antagonists have a weak affinity for the V_2R.

None of the 20 VP/OT analogs tested in our experiments displayed a better affinity for the human V_3R than the endogenous ligand VP itself (K_i = 1.1 nM). OT, the other endogenous ligand in humans, has a very low affinity for the V_3R (K_i = 1782 nM). The V_1R antagonist 4-OH-phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2, the most potent V_1R antagonist tested in CHO-V1 cells, also displays a high affinity for the human V_3R (K_i = 2.2 nM). Since this compound can be radioiodinated, it may replace tritiated VP as a radioligand with high specific activity to carry out further ligand binding characterization of the human V_3R. Recently, Barberis et al. (1995) reported that the same compound displays also a high affinity for the rat V_3R (K_i = 4.5 nM) and significantly blocks VP-induced ACTH release in cultured rat pituitary cells. The linear V_1/V_3 antagonist phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2, which displays the highest affinity for the porcine V_3R (K_i = 37.2 nM) among VP analogs recently available, also exhibits a reasonable affinity for the human V_3R in our studies (K_i = 16 nM); however it behaves as a more potent V_1R analog in both species. Similarly, deamino[1](D-3-(Pyridyl)Ala^2-Arg^8)vasopressin, which was reported to block the rat pituitary V_3R, displays 10-fold higher affinity for the human V_1R (K_i = 4.3 nM) than for the human V_3R (K_i = 50 nM). Finally, the three non peptide V_1R or V_2R antagonists tested in our cell lines have a low affinity for the human V_3R. The results in Table 1 indicate that no compound displays a higher affinity for the human V_3R than the native hormone VP itself and that none is selective for this receptor subtype. The availability of the CHO-V3 cells should facilitate the screening and development of such selective ligands.

Examination of the ligand binding profile of the CHO-OT cell line confirms that the two OT antagonists tested have an excellent affinity for the human OT receptor (K_i = 0.6 and 0.5 nM), but as noted above, their affinity for the human V_1R is also quite good (K_i = 3.9 and 7.6 nM). Among the V_1R antagonists, the affinity of the reference compound $d(CH_2)_5$Tyr(Me)VP for the human OTR appears to be quite high (K_i = 3 nM). In addition, several linear V_1R antagonists display a good affinity for the human OTR, including the V_1 antagonist 4OH-phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (K_i = 0.4 nM). Finally, the V_2 analogs and V_3 antagonists have a poor affinity for the human OT receptor.

This lack of selectivity of some compounds for the human VP/OT receptor subtypes, together with species-related differences (Kinter et al., 1993), may explain the discrepancies regarding the parti-

cipation of these subtypes in various signaling pathways. For instance, the vasodilatory action of pharmacologic doses of dDVP or dVDVP have been thought to arise from the activation of putative endothelial receptors of the V_2 subtype; however conflicting results with various antagonists tested may suggest the participation of other receptor subtypes (Katusic, 1992; Naitoh et al., 1993). These results underscore the need for developing specific and potent analogs interacting with the various human VP/OT receptor subtypes.

Stimulation of DNA synthesis by VP/OT in CHO cells expressing the human V_1R, V_2R, V_3R, or OTR

The V_1-vascular receptor has been shown to elicit mitogenic responses, including stimulation of DNA synthesis, expression of oncogenes, and cell proliferation. The influence of other VP/OT receptor subtypes on these parameters is not well known. To begin to unravel this issue, we measured thymidine uptake in CHO cells stably transfected with the VP V_1Rs (CHO-V1 cells, B_{max} = 12.4 pmol/mg of protein), V_2Rs (CHO-V2 cells, B_{max} = 25 pmol/mg of protein), V_3Rs (CHO-V3 cells, B_{max} = 17.9 pmol/mg of protein) and OTRs (CHO-OT cells, B_{max} = 22.6 pmol/mg of protein), as an index of mitogenic activity elicited by activation of a given VP/OT receptor subtype. Sub-confluent monolayer cultures of CHO-V_1, CHO-V_2, CHO-V_3 and CHO-OT cells were grown in 24-well plates to measure thymidine uptake in the presence of VP or OT as described previously (Teutsch et al., 1992; Bihoreau et al., 1993; Thibonnier et al., 1996). Cells were washed with 500 µl of F12 medium and grown for 72 h in 500 µl of F12 medium supplemented with 25 mM Hepes and 0.1% BSA. Cells were treated with increasing concentrations of VP or OT (ranging 10^{-12} to 10^{-6} M) for 18 h, followed by incubation with 10 µCi of [^{3}H]thymidine for 45 min. The cells were subsequently transferred on ice, washed twice with 0.5 ml of ice-cold PBS, fixed with 1 ml of ice-cold 10% TCA for 30 min at 4°C, washed twice with 1 ml of ice-cold 5% TCA solution, and solubilized with 250 µl of 0.1 N NaOH–0.1% SDS. Aliquots were collected and radioactivity was measured in a scintillation counter. Cells were stimulated with either increasing VP or OT concentrations (Fig. 1) or 10% fetal bovine serum. VP produced a dose-dependent increase of thymidine uptake in CHO-V1 (E_{max} = +334%, EC_{50} = 1.30 nM) and CHO-V3 cells (E_{max} = +284%, EC_{50} = 2.01 nM). In CHO-V2 cells transfected with the human V_2 receptor, which couples to adenylyl cyclase, VP triggered a reduction of thymidine uptake reaching its nadir at 0.1 nM VP. OT produced a dose-dependent increase of thymidine uptake in CHO-OT cells (E_{max} = +205%, EC_{50} = 1.31 nM). Treatment with 10% fetal calf serum produced a +390% to +490% increase over baseline in these transfected cell lines. These data indicate that stimulation of V_1Rs, V_3Rs by VP as well as OTRs by OT produces a mitogenic response whereas VP occupancy of V_2Rs leads to an antimitogenic response. For similar levels of expression of receptors, the mitogenic efficacy can be ranked as follows: V_1Rs > V_3Rs > OTRs.

The mitogenic action of the V_1R is a well established phenomenon which has been observed for various cells expressing this VP receptor subtype. The mitogenic property of VP via activation of the V_1R is concentration-dependent and is the most potent among the responses noted for the different

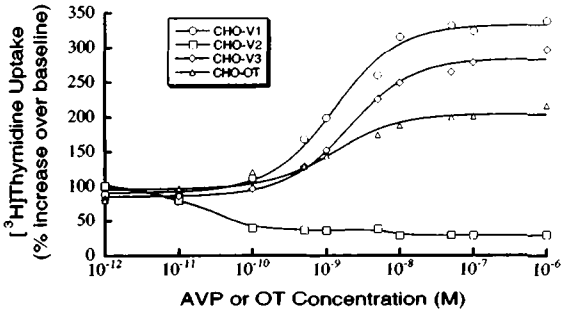


Fig. 1. Stimulation of DNA synthesis by VP in CHO cells transfected with V1, V2, V3 or OT receptors. Transfected CHO cells prepared as described in the Methods section were grown to confluence in 24-well dishes and incubated in serum-free F12 medium, pH 7.4 for 72 h. The cells were subsequently stimulated by increasing concentrations of VP or OT overnight, followed by incubation with [^{3}H]thymidine for 45 min, DNA precipitation and liquid scintillation counting. Results represent the average of three independent experiments carried out in octoplicates and are a revised and extended version of Fig. 4 in Thibonnier et al. (1997).

VP receptor subtypes. Assessment of VP-induced stimulation of thymidine uptake in cell lines transfected with a VP/OT receptor subtype will represent a convenient way to explore the mitogenic property of this specific subtype and test the influence of various pharmacological interventions.

The mitogenic action of the V_3R is a new finding which raises the issue of the role of V_3Rs in the development of ACTH-secreting tumors, as these tumors have been shown to express high levels of V_3Rs (Dahia et al., 1996; De Keyser et al., 1996).

The stimulation of thymidine uptake by OT in the CHO-OT cell line suggests that stimulation of OTRs also activates mitogenic signals. This finding deserves further attention because expression of the OTRs has been detected in human breast cancer tissue by immunohistochemistry and confirmed by Northern blotting and RT-PCR (Ito et al., 1996). By the same token, OT stimulates the proliferation of myoepithelial cells (Sapino et al., 1993). This mitogenic effect of OTRs stimulation should be examined, especially in the context of breast cancer.

The reduction of thymidine uptake by VP in the CHO-V2 cell line indicates that occupancy of V_2Rs by VP results in decreased cell proliferation whereas stimulation of V_1Rs, V_3Rs by VP and OTRs by OT produces a mitogenic response. This seems to suggest a negative correlation between VP-stimulated increase in cAMP levels and cell proliferation.

Modulation of cAMP production by VP/OT in CHO cells expressing the human V_1R, V_2R, V_3R, or OTR

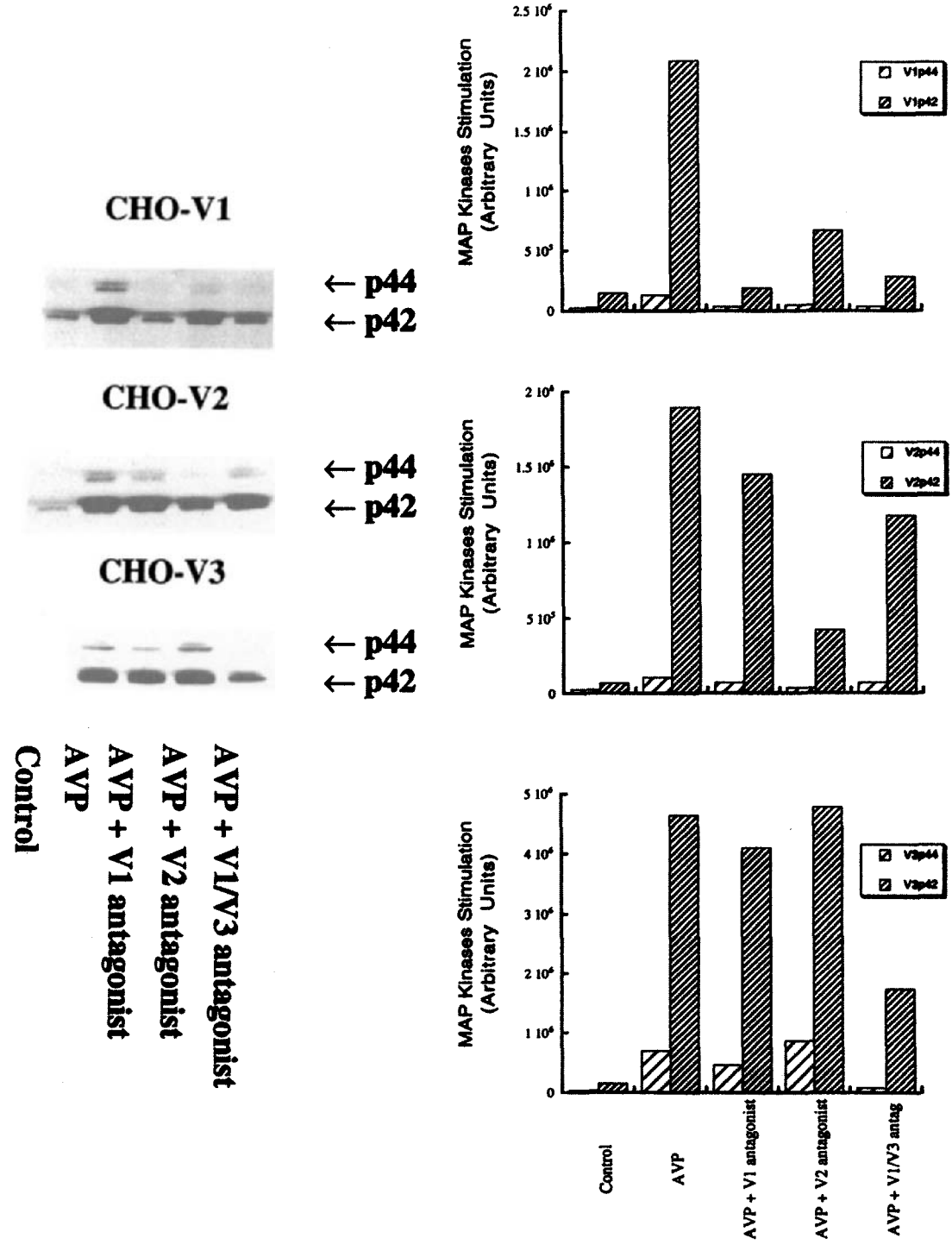
To examine the possible role of cAMP in the mechanisms underlying the effect of VP or OT on thymidine uptake in CHO cells transfected with the various VP/OT receptor subtypes, we directly measured VP- or OT-stimulated formation of cAMP in CHO cells expressing the V_1Rs, V_2Rs, V_3Rs or OTRs. Sub-confluent monolayer cultures of control and transfected CHO cells were grown for 48 h in 12-well dishes, serum-depleted in F12/HEPES media for 4 h, followed by labeling in media containing 1 μCi/ml of [^{3}H]adenine for 2 h. Cells were washed and incubated in media containing 0.5 mM IBMX, in the absence or presence of 10 μM forskolin and/or different amounts of VP for 10 min. The reaction was stopped by removal of media and addition of 0.5 ml ice-cold 5% TCA containing 1.5 mM cAMP. Separation of [^{3}H]cAMP was carried out as described by Evans et al., 1984). The amount of [^{3}H]cAMP synthesized during the incubation with agonists was expressed as a fraction of total labeled nucleotides present in the cell extracts (10^{-4} × cAMP dpm/total dpm in acid extract).

VP stimulation of CHO-V1 cells did not modify cAMP formation whereas forskolin did (5-fold increase). VP stimulation of CHO-V2 cells produced a 9-fold increase of cAMP formation, while forskolin alone and the combination of VP and forskolin produced respectively a 3-fold and a 20-fold increase of cAMP production. In CHO-V3 cells expressing levels of receptor density similar to those of the CHO-V1 and CHO-V2 cells, VP did not alter cAMP production. However, V_3R-driven stimulation of cAMP production was observed in cells expressing high levels of this receptor (Thibonnier et al., 1997). Finally, OT did not alter cAMP production in CHO-OT cells. These results suggest that stimulation of thymidine uptake by VP/OT receptors occurs only for the subtypes which do not stimulate cAMP production (V_1R, OT and low and medium levels of V_3Rs.

VP Stimulation of the MAP kinase pathway

The various VP receptor subtypes show a differential G protein coupling profile. The mitogen-activated protein kinases (MAPKs) are a point of convergence for mitogenic signals triggered by several classes of cell surface receptors including the GPCRs. G_i- and G_q- coupled receptors stimulate MAPK activation via distinct signaling pathways (Hawes et al., 1995). In transfected COS-7 cells, MAP kinases can be stimulated by G_s, G_i and G_q through participation of both α and $\beta\gamma$ subunits (Faure et al., 1994).

VP-dependent activation of MAP kinases was examined in CHO cells transfected with the various VP receptor subtypes. As activation of the human OTR by OT has been recently been shown to stimulate MAP kinase activity (Ohmichi et al., 1995), experiments with CHO-OT cell line were not


CHO-V1
← p44
← p42
CHO-V2
← p44
← p42
CHO-V3
← p44
← p42
Control
AVP
AVP + V1 antagonist
AVP + V2 antagonist
AVP + V1/V3 antagonist
MAP Kinases Stimulation (Arbitrary Units)
V1p44
V1p42
V2p44
V2p42
V3p44
V3p42
Control
AVP
AVP + V1 antagonist
AVP + V2 antagonist
AVP + V1/V3 antag

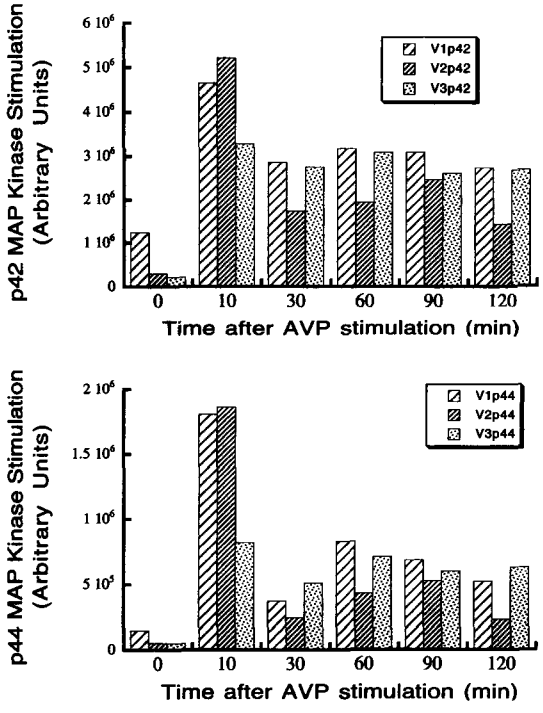


Fig. 3. Time course of phosphorylation of p42 and p44 MAP kinases induced by VP in CHO cells stably expressing human V1, V2, or V3 receptors. Transfected CHO cells prepared as described in the Methods section were grown to confluence in 12-well dishes and incubated in serum-free F12 medium (pH 7.4) for 72 h. The cells were stimulated by VP (10^{-7} M) for varying times and immunoblotting was performed with a phospho-specific p44/42 MAPK (Tyr204) antibody. Results represent a revised and extended version of Fig. 8 in Thibonnier et al. (1997).

performed. Phosphorylation of p42 and p44 MAP kinases by VP was measured in sub-confluent monolayer cultures of CHO-V_1, CHO-V_2 and CHO-V_3 cells grown in 12-well plates. Cells were serum-starved for 48 h before the experiments. After stimulation with VP alone or in the presence

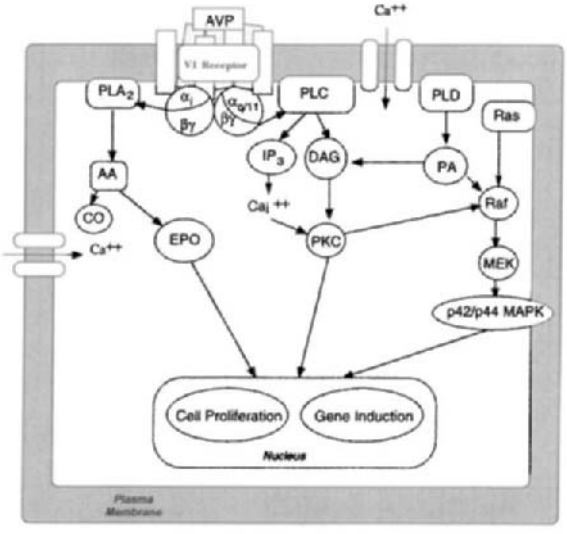


Fig. 4. Intracellular pathways coupled to the activation of the human VP V_1-vascular receptor. Abbreviations are: α, $\beta\gamma$, alpha subunits and beta-gamma dimers of G proteins; PLC, phospholipase C; PLD, phospholipase D; PLA_2, phospholipase A_2; IP_3, inositol-1,4,5 trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; AA, arachidonic acid; CO, cyclooxygenase; EPO, epoxygenase; PA, phosphatidic acid; ERK, extracellular signal-regulated kinase; MEK, mitogen activated ERK kinase; MAPK, mitogen activated protein kinase; cAMP, cyclic AMP; PKA, protein kinase A.

of antagonists, the cells were washed twice with ice-cold PBS and lyzed in buffer containing 50 mM Hepes-Tris (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 10 μg/ml pepstatin, 50 μg/ml bestatin, 200 μM Na_3PO_4, and 1 mM NaF. The lyzed cells were scrapped and centrifuged at 14 000 × g for 20 min. The supernatants were subjected to SDS-PAGE. After electrophoresis, proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA) and immunoblotted overnight at

Fig. 2. VP-induced phosphorylation of p42 and p44 MAP kinases in CHO cells stably expressing human V1, V2, or V3 receptors. Transfected CHO-V3High cells prepared as described in the Methods section were grown to confluence in 12-well dishes and incubated in serum-free F12 medium (pH 7.4) for 72 h. The cells were stimulated for 10 min by VP alone (0.1 μM) or in the presence of the V_1 antagonist $d(CH_2)_5Tyr(Me)VP$ or the V_2 antagonist $d(CH_2)_5[D\text{-}Ile^2\text{-}Ile^4\text{-}Ala\text{-}NH_2]VP$ or the V_1/V_3 antagonist Phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2 (0.1 μM). Immunoblotting was performed with a phospho-specific p44/42 MAPK (Tyr204) antibody. Both the immunoblots (left panels) and their corresponding densitometries (right panels) are shown.

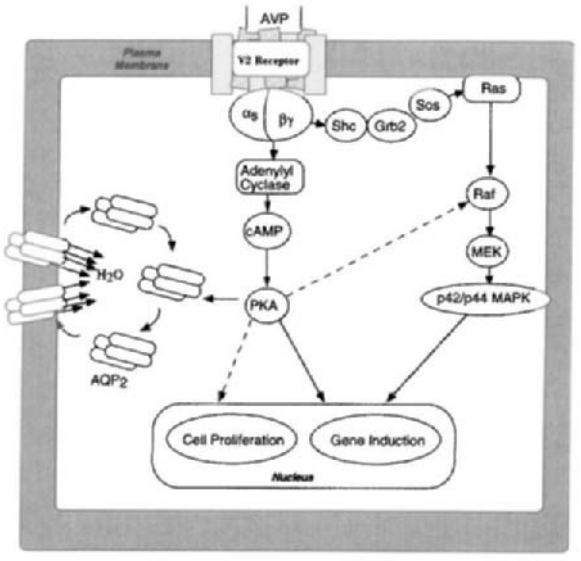


Fig. 5. Intracellular pathways coupled to the activation of the human VP V_2-renal receptor. For abbreviations, see legend of Fig. 4; AQP_2, aquaporin in two channels.

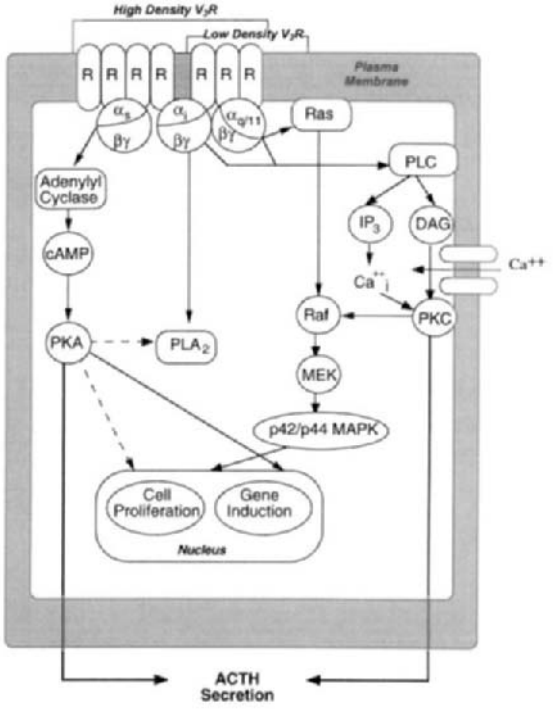


Fig. 6. Intracellular pathways coupled to the activation of the human VP V_3-pituitary receptor. For abbreviations, see legend of Fig. 4. This figure is derived from Fig. 10 in Thibonnier et al. (1997).

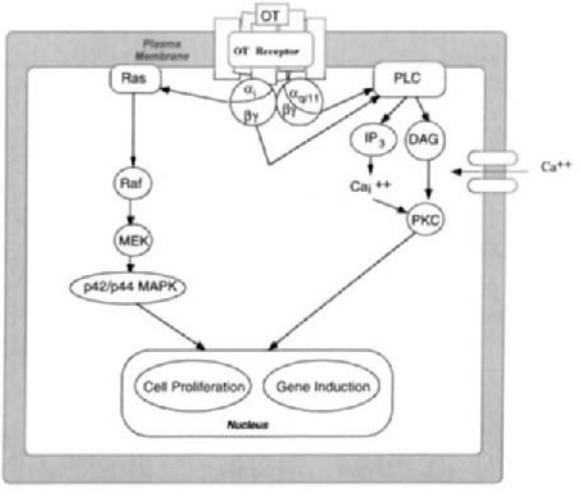


Fig. 7. Intracellular pathways coupled to the activation of the human Oxytocin receptor. For abbreviations, see legend of Fig. 4.

4°C with the phospho-specific p44/42 MAPK (Tyr204) antibody (rabbit polyclonal IgG affinity purified antibody from New England Biolabs, Beverly, MA). Immunodetection was carried out with the ECL kit (Amersham, Arlington Heights, IL) following the manufacturer's recommendations. Quantification of MAP kinases phosphorylation was performed by scanning densitometry of the autoradiograms with a USB SciScan 5000 automated scanning system from USB (Cleveland, OH). Fig. 2 shows the immunoblotting with a phospho-specific p44/42 MAPK (Tyr204) antibody of CHO-V1, CHO-V2 and CHO-V3 cells stimulated by VP. VP stimulation (0.1 μM) for 10 min produced a significant increase in phosphorylation of p42 and p44 MAP kinases. In the CHO-V1 cells, this effect was potently blocked by an equimolar concentration of the V_1 antagonist $d(CH_2)_5$Tyr-(Me)VP. The V_2 antagonist $d(CH_2)_5$[D-Ile^2-Ile^4-Ala-NH_2]VP and the V_1/V_3 antagonist phenyl-acetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2, which are in fact non-selective analogs binding to the V_1 receptor, did reduce the effect of VP on MAP kinases phosphorylation in CHO-V1 cells. In CHO-V2 cells, VP also stimulated MAP kinases phosphorylation, an effect that was specifically blocked

by the V_2 antagonist $d(CH_2)_5[D\text{-}Ile^2\text{-}Ile^4\text{-}Ala\text{-}NH_2]VP$. The V_1 antagonist $d(CH_2)_5Tyr(Me)VP$ and the V_1/V_3 antagonist phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2 did not alter VP effect on MAP kinase in CHO-V2 cells. Finally, VP stimulation of MAP kinase phosphorylation occurred in CHO-V3 cells and was specifically blocked by the V_1/V_3 antagonist phenylacetyl-D-Tyr-Phe-Val-Asn-Arg-Pro-Arg-Arg-NH_2, whereas the V_1 and V_2 antagonists were ineffective.

The extent and duration of VP-induced phosphorylation of p42 and p44 MAP kinases were explored in CHO cells stably expressing V_1Rs, V_2Rs, or V_3Rs (Fig. 3). The phosphorylation of p42 and p44 induced by VP stimulation (100 nM) peaked at 10 minutes and started to decay slowly afterwards in all cell types. In these transfected CHO cells, the effect of VP lasted for at least 2 h.

Stimulation of MAP kinase phosphorylation by the various types of VP receptors suggests that different pathways are involved in the process. The V_1Rs presumably act through activation of $G_{q/11}$, PLC, PKC and PI_3Kinase (Nishioka et al., 1995; Thibonnier et al., 1997). Activation of MAP kinase phosphorylation by the V_2R, presumably through activation of G_s, is interesting in view of the reported inhibition of MAP kinases by cAMP (Faure et al., 1994). The role of G_s in regulating cell growth and differentiation is complex and opposing effects have been proposed (Crespo et al., 1995): activation of MAP kinase by the $\beta\gamma$-subunit of G_s and inhibition of MAP kinase by cAMP-dependent protein kinase activation following G_s-dependent activation of adenylyl cyclase. The V_3Rs presumably activate the MAP kinase pathway through activation of $G_{q/11}$ in a PLC- and PKC-dependent fashion (Thibonnier et al., 1997). The OTRs have been shown to activate MAP kinase through activation of G_i in a pertussis toxin-sensitive manner (Ohmichi et al., 1995). The different intracellular signal pathways linked to activation of the V_1-vascular, V_2-renal, V_3-pituitary and oxytocin receptors are depicted in Figs. 4–7.

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CHAPTER 3.1.2

Function and molecular basis of action of vasopressin 4–8 and its analogues in rat brain

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VP 4–8 as a highly potent behavioral-active metabolite of arginine-vasopressin (VP) has been studied in detail at four levels, i.e. ligand level, membrane binding level, intracellular level and nuclear level. The purpose of this chapter is to review and discuss the main results obtained from our recent pharmacological and biochemical investigations which are described as follows: 1, structure–function relationship of VP 4–8 and its analogs; 2, some characters of VP 4–8-specific binding, the distribution of the binding sites in the rat brain and the consequent effect on long-term potentiation of synaptic transmission; 3, a putative receptor-mediated signaling pathway involving second messenger IP_3, immediately-early gene c-*fos* transcription and protein kinase PKC, CaMKII and MAPK; 4, peptide-induced enhancement of some crucial functional proteins such as calmodulin, nerve growth factor (NGF) and brain-derived nerve growth factor (BDNF). The physiological significance of the events following VP 4–8 administration and particularly, its possible role in learning and memory processes are discussed.

Introduction

The neurohypophyseal hormone arginine-vasopressin (VP) plays important roles in the peripheral as well as in the central nervous system. VP acts as a neural regulator via the receptors located in the peripheral as well as in the CNS. In the CNS, both V_{1a} and V_{1b} receptors coupled to G proteins affect the inositol phospholipid metabolism and adjust the intracellular calcium levels (Kirk et al., 1981; Shewey and Dorsa, 1988). Receptor V_{1a} also exists in vasocardial regions and plays the role of vasoconstrictor (Aiyar et al., 1986). V_2 receptor located in kidney has effects on the antidiuretic process (Butlen et al., 1978; Guillon et al., 1982; Dorso et al., 1983).

Burbach and Lebouille (1983) and Burbach et al. (1983) reported that incubation of VP with brain synaptic membrane resulted in the release of a series of C-terminal fragments of VP and experiments with ^{14}C-labelled VP suggested that it was due to an aminopeptidase-like enzyme. Burbach et al. (1987) further determined the aminopeptidase activities by immunological and chromatographic techniques and identified the endogenous presence of VP fragments in the brain. Several avoidance behavior tests had been used to determine the behavioral potency of VP fragments administered via different routes. Abundant experimental data (Burbach and Lebouille, 1983; Burbach et al., 1983; De Wied et al., 1984a,b, 1991; de Jong et al., 1985; Gaffori and De Wied, 1986) suggested that these fragments had stronger effects in behavioral response than the parent peptide. These fragments not only increased the resistance to the extinction of active avoidance behavior but also facilitated the consolidation and retrieval of passive avoidance behavioral response although the potencies usually differed from each other in the different tests (Gaffori and De Wied, 1986; Kovacs et al., 1986; De Wied et al., 1987). These effects were dose-dependent and had a long term nature (De Wied et al., 1987; Beckwith et al., 1990).

Although Le Moal et al. (1981) believed that VP acted mainly at peripheral receptor systems, thus altering visceral afferent impulses which would influence behavior, experiments with VP and VP

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4–8 (Burbach et al., 1983; De Wied et al., 1984a,b, 1991; de Jong et al., 1985; Zlokovic et al., 1992) indicated that the peripheral effects and the behavioral actions were separated. They were mediated by different receptor systems. The receptors involved in behavioral actions were likely present in the central nervous system, whereas those mediating the peripheral effects were mainly located in the periphery. The peripheral receptor systems could not be activated by VP 4–8 and thus could hardly be involved in mediating the behavioral effects of this peptide. From these data, Burbach et al. concluded that VP served as a precursor of the more active neuropeptide VP 4–8 which mediated the central function of its parent peptide and was probably related to specific receptor or important endogenous molecule in CNS to modulate behavioral processes (Burbach and Lebouille, 1983; De Wied et al., 1984a). It has been proposed that this peptide should be used in further studies on the central effects of neurohypophyseal hormones (De Wied et al., 1984b).

In our laboratory, a series of studies on VP 4–8 and its synthetic analogs has been done not only on a behavioral level but also on physiological and biochemical levels in the rat brain. The purpose of this chapter is to review and discuss the main results of pharmacological and biochemical studies, including structure–function relationships of VP 4–8, characterization of its binding sites in CNS, a putative branching signaling pathway, the enhancement of neurotrophin gene expression and so on. Based on these experiments, it is suggested that VP 4–8 and its active analogs may share a specific receptor mediating signaling pathways in rat limbic system and eventually affect long-term memory by expression of neurotrophin genes.

Structure–activity relationship studies

In an attempt to explore the physiological significance of VP in CNS on the molecular level, VP analogs were synthesized and their behavioral potencies were evaluated. In these experiments (Chen et al., 1988; Liu and Lin, 1990), brightness discrimination (BD) and the shuttle box test were used as parameters to measure learning and memory potency of these peptides. Considering serious uncomfortable responses might be caused by drug administration, the long-term learning and memory effects of hypertonic saline was first observed (Chen et al., 1988) before the two analogs: 1-desamino-8-D-vasopressin (DDVP) and 9-desglycylamide-DDVP (DGDDVP) were tested. Chen et al. found that neonatal treatment with DDVP and hypertonic saline both facilitated the acquisition and subsequent maintenance of brightness discrimination in immature and mature rats, and post-treatment with DDVP also enhanced the retention of BD in adult rats. It is interpreted that VP is released by the administration of hypertonic saline; thus both endogenous VP and exogenous DDVP facilitate remarkably the learning processes and inhibit the extinction of memory.

Since the short analogs of VP are more active in the memory process than VP itself, it is interesting to study the structure–activity relationship of VP 4–8. A comparison between VP 4–8 and its analogs, such as its cysteinyl methyl ester ([Cyt^6-OMe]VP 4–8) and their D-arginine analogs: [D-Arg]-VP 4–8 (DVP 4–8) and [$D\text{-}Arg^8$,Cyt^6-OMe]VP 4–8 (Lin and Liu, 1990; Liu and Lin, 1990) showed that, unlike VP, both VP 4–8 and [Cyt^6-OMe]VP 4–8 are more potent than their D-Arg isomers in the behavioral response either in newborn rats or in adult rats. The differential effects of D-Arg in the long and short analogs on the behavioral response implies that VP 4–8 specific receptors are sensitive to the conformation of the peptide. 2D-NMR studies (Xu et al., 1990) indicated that VP 4–8 had a compact structure, in which the side chains of Asn^5 and Arg^8 might form a hydrogen bond as shown in Fig. 1. Since D-arginine substitution in VP 4–8 results in a dramatic decrease of potency, such a compact structure with a sequence motif -[L-Asn]-xxx-xxx-[L-Arg] is necessary for the behavioral activity. Further molecular dynamics analysis showed that residue $pGlu^4$ was highly flexible and deletion of $pGlu^4$ and/or substitution of Cyt^6 with hydrophobic residues would not attenuate the behavior potency (Wang et al., 1992, 1994).

To understand more on the structure–activity relationship, the roles of all residues in VP 4–8 were investigated by searching 45 homologs with four or five amino acid residues and analogs were designed according to the predicted conformational

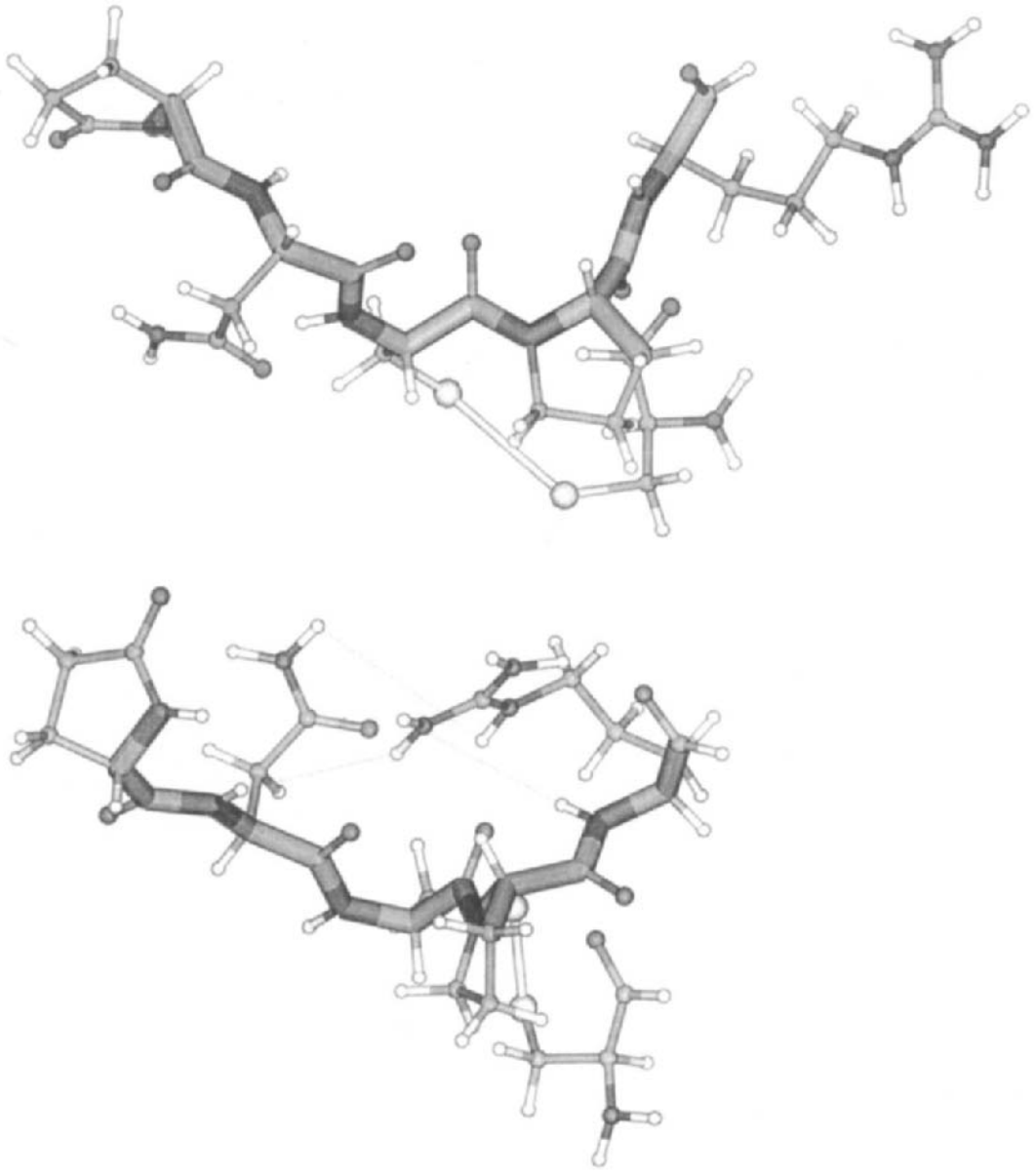

Fig. 1. Structure simulation of VP 4–8 (down) and [D-Arg^8]VP 4–8 (up).

properties. Experimental results from passive avoidance behavior tests using synthetic analogs showed good agreement with theoretical calculations, for example, pGlu-Asp-Cyt-Pro-Arg (ZDC(C)PR) was found to be an effective antagonist of VP 4–8, indicating that the compact structure with a certain flexibility and the third and fourth hydrophobic residues in VP 4–8 molecule were important for recognizing its receptor (Wang et al., 1994).

This conclusion may help us to design potent agonist and antagonist of this new neuropeptide.

Currently, tetrapeptide Asn-Leu-Pro-Arg (NLPR) has been found to be a potential drug candidate for curing memory impairment, oral administration of it in memory-impaired rat resulted not only in improving the acquisition and maintenance of behavioral response (BD), but also in facilitating significantly NGF expression in the brain (Zhou et al., 1995b).

Physiological and neurochemical studies

Binding sites in the rat brain

Based on the data available so far, we put forward the hypothesis that there were specific receptor systems for VP 4–8 in central nervous system, through which this neuropeptide exerts its functions on learning and memory processes. VP 4–8 specific binding sites in the rat brain has been shown by using ^{35}S-labelled VP 4–8 with high specific activity (280 Ci/mmol) (Du et al., 1994a,b). Density-scanning on radiophotogram of rat brain slices showed that specific binding sites of VP 4–8 were present in many brain areas, such as amygdala, anterior cortex, hippocampus and so on. Further analysis by observing the selective damages of hippocampal neurons by neurotoxins, kainic acid or colchicine, showed that the binding sites in rat hippocampus were present on the whole hippocampal pyramidal cell layer and granular cell layer of gyrus (Du et al., 1994a). It is worthy to note that the biological functions of hippocampus are closely related to learning and memory. However, the binding sites differed from the molecular layer and hilus of dentate gyrus reported for VP or VP 4–9 (De Kloet et al., 1985; Pearlmutter et al., 1985; Brinton et al., 1986).

The above findings led us to undertake a more detailed investigation on the characteristics of VP 4–8-specific binding in the CNS. Results from radio-binding assay (RRA) (Du et al., 1994b) demonstrated that VP 4–8 had a tight and specific binding to the rat anterior cortical and hippocampal synaptosomal membranes, and had a wide range regional distribution in the rat brain, especially in the limbic system. The specific binding of VP 4–8 on cortical membranes was saturable and reversible with only one binding site of high affinity, having a K_d value of 3.12 or 4.41 nM, and a B_{max} value of 31 or 21 fmol/mg protein in the presence of Ni^{2+} or Mg^{2+} respectively. It seems significantly different from the reported properties of VP binding sites on brain membranes at pH 7.4 (Pearlmutter et al., 1985). Firstly, VP had two classes of binding sites on the synaptosomal membranes, with K_d values of 0.54 and 16 nM and B_{max} values of 5.0 and 47 fmol/mg protein (Junig et al., 1985), and none of them were close to those of VP 4–8. Secondly, the enhancement by nickel salts on VP binding (almost 10 times) (Pearlmutter et al., 1983; Junig et al., 1985) was much larger than that on VP 4–8 binding (no more than two times). Thirdly, the time for reaching equilibrium we observed in the VP 4–8 binding process was only one-third of that for VP. Fourthly, the regional distribution of VP binding sites in rat brain was (Pearlmutter et al., 1983) similar to that found for VP 4–8 binding, but a detailed analysis of binding sites in hippocampus revealed a substantial difference between VP and its metabolites on the location of their binding sites (Brinton et al., 1984, 1986; Du et al., 1994a,b; Petracca et al., 1986). Finally, it was found that VP did not show detectable competition with the VP 4–8 binding even at a 1000 molar ratio (Du et al., 1994b).

Furthermore, the VP 4–8 binding sites in new born rat brain could be induced by postnatal s.c. administration of 0.2 μg of VP 4–8 per animal (Du et al., 1994a), through the acceleration of the hippocampus development or the modulation of a special receptor biosynthesis.

Ma et al. (pers. commun.) recently found that tetrapeptide NLPR, as a potent agonist of VP 4–8, showed a tight and specific binding on rat anterior cortical synaptic membranes with K_d 1.6 nM and B_{max} 11.2 fmol/mg protein in the presence of 5 mM $NiCl_2$ (pH 7.4) and this binding was almost totally blocked by ZDC(C)PR, an antagonist of VP 4–8, suggesting that NLPR and VP 4–8 might share a common receptor in the cell membrane.

Potentiating synaptic transmission

How does VP 4–8 affect memory responses after its binding to rat hippocampus and cortex? Are there any subsequent cellular events? To answer these question, Rong et al. (1993) studied the

effects of VP 4–8 on the synaptic transmission in rat hippocampus slices, at both extracellular and intracellular levels as long-term potentiation (LTP) is thought to underlie certain forms of learning and memory. They found that 0.5 μM to 10 nM of VP 4–8 dramatically stimulated the excitatory postsynaptic potential (EPSP) in the CA1 region of Schaffer collateral/commissural fibers. After incubation for 15 min or more, the EPSP became action potential (intracellular). Similar stimulation was also found in the extracellular level as 0.2 μM of VP 4–8 increased the amplitude of field EPSP evoked in the CA1 region by low frequency stimulation (0.1 Hz) and after washing the potentiation effect was sustained, at least for as long as the recording lasted (3.5 h). Furthermore, the effect of VP 4–8 on LTP of synaptic transmission was observed by brief high frequency stimulation of afferent fibers in two slices of the same hippocampus simultaneously perfused with standard medium containing or not containing the peptide as sample or control. The enhancing effect of 0.2 μM of VP 4–8 on the amplitude of LTP of the CA1 region of the slice was significantly larger than that of the control by 50–100% and the increase in amplitude was long lasting after washing.

The dose-dependent effect of VP 4–8 on the amplitude of pEPSP in CA1 region was tested by using different peptide concentrations (Rong et al., 1993). The threshold concentration for generating the effect was 1×10^{12} M. We tested the effect of arginine vasopressin (VP) and found that the lowest effective concentration of VP was usually in the range of 1×10^{-8} M to 10^{9} M, indicating that the enhancing potency of VP 4–8 was thousands times higher than that of VP. A clear parallel relation existed in the behavioral potencies of these two peptides, as has been found in passive avoidance trials on adult rats where VP 4–8 is a hundred or thousand times more effective than its parent molecule VP (Burbach and Lebouille, 1983; Burbach et al., 1983; Kovacs et al., 1986; Gaffori and De Wied, 1986; Lin and Liu, 1990; Liu and Lin, 1990). This supports the postulation that the effect of VP on hippocampal pyramidal neurones is not due to VP itself, but to its metabolites, e.g. VP 4–8.

The potentiation of synaptic transmission by VP 4–8 in mossy fiber-CA3 pyramidal neuron synapse (Rong et al., 1993) was similar to that in Schaffer collateral/commissural fiber synapse. Since NMDA-receptor is not included in the transmission of mossy fiber-CA3, the way for VP 4–8 stimulating LTP may not be through the NMDA-receptor. Additional evidence is that: D,L-APV, an NMDA-receptor antagonist, completely blocked the LTP following tetanic stimulation, but did not block the potentiation effect of VP 4–8. In general, the binding of VP 4–8 potentiates the synaptic transmission in hippocampus long-term and this potentiation is mainly due to a NMDA-plus mechanism.

Receptor-mediated signaling pathway

Stimulation of GTP-binding protein

By using ^{35}S-labelled GTPγS Yan et al. recently found that VP 4–8 dose-dependently stimulated [^{35}S]GTPγS binding on hippocampal synaptic membranes and this stimulation was blocked by pertussis toxin (PTX), a selective inhibitor of the G-protein-coupled receptor (GPCR), and by the antagonist ZDC(C)PR. Since PTX and ZDC(C)PR can respectively block the VP 4–8 signaling pathway at the receptor and the GP level, it is suggested that the VP 4–8 receptor may belong to a GPCR family (Yan et al., 1998).

Accumulation of inositol triphosphates

It is well known that the signal transduction pathway of inositol phospholipid metabolism exists in the nervous system and the binding sites for inositol 1,4,5-triphosphate (IP_3) are more abundant in brain than in peripheral tissues (Worley et al., 1987). Berridge (1986) indicated that the signal transduction caused by inositol phosphate was responsible for the acquisition of long-term memory. Therefore, the question is whether a certain neurotransmitter that improves learning and memory behavior of animals can also stimulate IP_3 signal transduction in the hippocampus. It was reported that VP could affect the inositol phospholipid metabolism in the rat brain (Stephens and Logan, 1986), so the effects of VP 4–8 and their analogs on the accumulation of IP_3 were studied (Gu and Du, 1991a). In the presence of GTP, VP 4–8 could markedly

stimulate inositol phospholipid metabolism in the hippocampal tissue slices in vitro, and the stimulation increased as the incubation time was prolonged and reached a maximum at about 30 min. Under the same conditions, no detectable changes of cAMP concentration could be observed. Studies on the effect of VP analogs, including DDVP, DGDDVP, DVP 4–8 and [D-Arg^8, Cyt^{6}OMe]VP 4–8, on enhancing the accumulation of IP_3 revealed that all these effects were dose-dependent but their enhancing potencies were very different from one another. Based on the dose-response curves, the half-effective dosage, EC_{50}, was found to be 1×10^{-8} M for VP 4–8 while it was 3.3×10^{-6} M for VP and 5×10^{-6} M to 10^{-5} M for the others (see Table 1). This means that the analogs, except VP 4–8, have potencies at the same level as VP (about 1–10 μM) and a hundred times lower than that of VP 4–8. This order of difference is consistent with the differences in behavioral activity and LTP enhancement as previously described. This result further supports the concept that VP acts as a precursor of a new neuropeptide in the brain and the behavioral effects of VP 4–8 are mediated by receptor binding and subsequent IP_3 accumulation.

Growth-associate protein (GAP43/B50) is a main substrate of protein kinase C (PKC) in brain cell membranes and may be functionally related to synaptic plasticity, axon elongation and LTP. An increase in the concentrations of IP_3 and 1,2-diacylglycerol (DG) following VP 4–8 administration readily activated PKC and enhanced GAP43 phosphorylation. Considering VP 4–8 enhanced LTP in pyramidal neurones, it is rational to postulate that the LTP effect of VP 4–8 is at least partly mediated by the phosphorylation of GAP43 through altering the plasticity of synapse and consequently releasing calmodulin (CaM) from the GAP43CaM complex. In fact, significant acceleration of phosphorylated GAP43 maturation was found in the early developmental stage of newborn rats (4–16 days old) by comparing the postnatal VP 4–8 administered group with the control group (Chen et al., 1993) and Ca^{2+}/CaM-dependent protein kinase II (CaMKII) in adult rat brain was readily activated following VP 4–8 administration (Qiao et al., 1998) which is discussed in detail below.

It is well known that VP acts on two distinct types of receptors in the periphery (Creba et al., 1983): V_1-receptors, by which VP stimulates the IP_3 system in the epithelial cells of blood vessels and V_2-receptors, by which VP stimulates the activity of adenylcyclase in renal tubule cells. The receptor of VP in hippocampal cells belongs mainly to V_1-type, but VP 4–8 cannot be recognized by V_1-receptor expressed in frog eggs (Du et al., unpublished work). It is believed that this receptor in the brain is different from that of epithelial cells of blood vessels and may be a new subtype or special binding state of V_1.

Table 1
The concentration of agonists for half-maximal IP_3 accumulation

Peptide	EC_{50} (M)
ZNC(C)PR	1.0×10^{-8}
VP	3.3×10^{-6}
DDVP	5.0×10^{-6}
DDDGVP	7.5×10^{-6}
[D-Arg^8]-VP 4–8	1.2×10^{-5}
[Cyt^6-OMe, D-Arg^8]-VP 4–8	Weak
OT	ND

Activation of Ca^{2+}/CaM-dependent kinase II

Calmodulin (CaM) is a ubiquitous intracellular calcium receptor which can be released from CaM-binding proteins such as GAP43 and MARCKS when they are phosphorylated by PKC (Chen et al., 1993). Effective CaM is responsible for activating more than 20 enzymes (Olwin and Storm, 1985). Among them, Ca^{2+}/CaM-dependent kinase II (CaMKII) is a crucial enzyme for the formation of LTP. After Ca^{2+}/CaM-dependent autophosphorylation, CaMKII displays its protein kinase activity by switching the molecular conformation into an 'on' state. It has been found that LTP can be promptly induced by VP 4–8 in rat hippocampal slices, but the process of LTP is not related to the activation of NMDA receptor as described above. So it is interesting to observe the changes of effective CaM level and CaMKII activity in the brain of rats administered with VP 4–8. The level of CaMKII autophosphorylation in brain cytosolic

extracts was determined in the presence of Ca^{2+} by an enzymatic method (Qiao et al., 1998). A maximal increase of 2-fold in the cerebral cortex was observed 1 h after VP 4–8 administration, while 40% increase was found in the hippocampus. CaMKII activity represented by the level of its autophosphorylation appeared to be dependent on the concentrations of Ca^{2+} and CaM. Effective CaM level and its gene expression were separately analyzed by enzymatic determination and by Northern blot assay. It was depicted that in 1 h after VP 4–8 administration the effective CaM in plasma increased to 3.4-fold, while the level of CaM mRNA had no detectable alteration but 3 h later the CaM transcription increased significantly to a maximum of 2.5-fold and the protein level increased to 5.8-fold. This means that the increase of CaM may occur at two levels, i.e., delivery from the CaM pool (GAP43) and expression of the CaM gene, and the latter is not involved in the first stage of the peptide-induced LTP (within 0.5 h).

Since the enhancement of CaM expression may replenish the CaM pool, the sustained activation of CaMKII will lead to the enhancement of synaptic transmitter release and maintenance of the long-lasting LTP phenomena. Therefore, the involvement of both CaM and CaMKII in the peptide-induced LTP and temporary memory formation process was suggested.

Activation of MAPK and PKC

Mitogen-activated protein kinase (MAPK) has crucial functions in the multiple signaling pathway. It can be activated by a variety of extracellular stimuli mediated by respective receptors. In order to investigate its role in the signaling process evoked by VP 4–8, alteration of MAPK activities in the rat brain was analyzed by measuring its content and activity and using myelin basic protein (MBP) as a specific substrate (Qiao and Du, 1997). It was found that the s.c. administration of VP 4–8 resulted in a remarkable increase of MAPK activity in the hippocampus after 2 h and a slight increase in the cerebral cortex after 1 h. However, immunoblotting assay indicated that the activated MAPK was a 44 kDa molecule and its protein level had no detectable difference between the administration groups and the control, indicating that the increase of MAPK activity stimulated by VP 4–8 was caused by a short-term activation process through protein phosphorylation but not by protein expression.

Qiao et al. found that polymixin B (PMB), a PKC inhibitor, dramatically inhibited the peptide-activation of MAPK but had no influence on CaMKII activation, and KN-62, a specific inhibitor of CaMKII, totally blocked the activation of CaMKII by VP 4–8 but had no effect on the MAPK stimulation. Furthermore, the VP 4–8-stimulation of MAPK and CaMKII was both suppressed by an antagonist ZDC(C)PR and PTX but VP-stimulation of MAPK activity could not be blocked by PTX. In view of these facts, i.e., VP 4–8 binding cannot be competed by VP (Du et al., 1994b) and VP/OT receptors in the brain do not display affinity for VP 4–8 (De Kloet et al., 1985), we believe that the receptor for VP 4–8 may be a G_0-coupled receptor and the IP_3-associated VP 4–8 signal transduction is a branching pathway: one to CaM → CaMKII → LTP and another to PKC → MAPK → gene expression (Qiao and Du, 1996; Yan et al., 1998; Qiao et al., 1998).

Enhancement of c-fos expression

Although the function of *c-fos* is not yet clear, it has been suggested that it acts as a 'third messenger' molecule in signal transduction systems to couple short-term intracellular stimuli with long-term responses by altering gene expression (Curran and Morgan, 1987). For instance, under the stimulation of growth factors or neurotransmitters, *c-fos* expressed transiently in neurons (Sager et al., 1988). Expression of *c-fos* is important for synaptic differentiation which may be involved in neuroadaptive processes such as learning and memory (Greenberg and Ziff, 1986).

As mentioned previously, the effect of VP 4–8 on behavioral responses in rats was more potent than VP itself and was 300-fold more effective than VP to induce LTP in rat hippocampal slices. To explore the way through which the long-term memory processes occur, Gu and Du investigated the effect of VP 4–8 on *c-fos* and c-*src* transcription in the rat hippocampus. The results from Northern analysis showed that the maximal levels of *c-fos* and c-*src*

transcription in the hippocampus of new born rats were age-dependent and the peak times for *c-fos* and c-*src* were on day 9 and day 20, respectively. The mRNA levels were significantly increased by postnatal administration (s.c. 10 ng/day for 4 days) of VP 4–8 to 5-fold at day 9 for *c-fos* and 3–8-fold at day 20 for *c-src* versus saline groups (Gu and Du, 1991b). It is notable that although VP 4–8 markedly promoted the transcription of these oncogenes, it did not interrupt the developmental phases of the brain in the new born rat. Structure–activity studies showed that two behavioral inactive analogs, ZDC(C)PR and $(ZNCPR)_2$ both had a suppressive effect on the peptide stimulation of the cellular oncogene *c-fos* transcription in infant rats as shown in Table 2.

Zhou et al. (1995a) further found that the VP 4–8-induced enhancement of *c-fos* transcription was also detectable in adult rat hippocampal slices by the gel-mobility-shift method using a ^{32}P-labelled double-stranded oligonucleotide probe. Autoradiographic results showed that the expressed protein in the specific hybrid band was identified as Fos by anti-Fos antibody and that the content of Fos protein from the slices pretreated with 87 nM VP 4–8 was higher than those from the control. The peptide-induced increase of in vitro expression of *c-fos* gene reached a maximum (1.8-fold versus control group) by 1.5 h (Zhou et al., 1995a). Although an increase in *c-fos* mRNA may result from injury caused by direct injections into the brain (Morgan et al., 1987), the observed increases are specific receptor-mediated events, which is also supported by the phenomenon that the response to VP 4–8 was blocked by its antagonist ZDC(C)PR. It has been reported that a biochemical factor involved in the enhancement of *c-fos* expression in certain cells is the increase in intracellular calcium level (Morgan and Curran, 1986). As VP 4–8 can stimulate the in situ phospholipid metabolism and activate Ca^{2+}/CaM-dependent protein kinase II activity via a subtype of V_1-like receptor in rat brain (Gu and Du, 1991a), it is rational to suggest that the increase in *c-fos* expression in the brain may be a key factor in mediating the peptide effects on long-term memory by inducing some functional gene expression.

Table 2
Effects of VP 4–8 and its analogs on *c-fos* transcription in 9-day-old rat hippocampus[a]

Peptide (ng/rat)	Ratio of *c-fos* mRNA versus saline group
Saline	100
VP 4–8 (ZNC(C)PR) (10)	601 (582–620)
ZDC(C)PR (2)	66.2 (65.8–66.7)
$(ZNCPR)_2$, (2)	37.8 (37.5–38.0)
VP 4–8 (10) + ZDC(C)PR (2)	94.0 (80.0–106)

[a] Newborn rats were s.c. administered with peptide in 20 μl saline once per day for 4 days.

Nuclear events affected by VP 4–8

As mentioned above, VP 4–8 evoked a series of physiological and biochemical events in the rat brain such as potentiating synaptic transmission, enhancing the accumulation of second messenger IP_3 in hippocampal slices, accelerating the maturation of phosphoprotein B50/GAP43 and inducing *c-fos* protooncogene transcription in infant rat brain. These findings provide a molecular basis good enough for the short-term memory-enhancing effect of VP 4–8, but not enough for its long-term effect. Obviously, the enhancement of long-term memory requires alterations in gene expression.

In fact, the expression of a series of genes related to memory process has been reported; among them, neurotrophin genes are prominent in the formation of long-term memory and recovery from some memory dysfunctions (Olson, 1993).

Nerve growth factor gene expression

It is well known that protein Fos can bind Jun, a product of another protooncogene, to form a heterodimer which can bind to the AP1 site located in the promoter of genes encoding functional proteins to enhance the expression of these genes. The AP1 sequence was found to be involved in the promoter region of NGF gene (Scott et al., 1983), and the lesion-induced increase of NGF mRNA in rat nerve pieces was mediated by immediate-early gene *c-fos* (Hengerer et al., 1990). In view of the key position of neurotrophins in the formation of

long-term memory, it is interesting to see whether VP 4–8 has any effect on the expression of neurotrophin genes in the rat brain via receptor-binding and *c-fos* expression.

Zhou et al. (1995a, 1996) have studied the effect of exogenous VP 4–8 on the in vivo transcription of NGF gene in the rat brain by using quantitative Northern blot analysis with cDNA and antisense RNA probes to evaluate the enhancement of mRNA level and by using ELISA to demonstrate the increase of NGF protein. They found that, at both mRNA and protein levels, NGF expressions in the hippocampus and the cerebral cortex were significantly enhanced up to 3.1- and 6.4-fold in a time period of 12 h after s.c. administration of 0.2 μg VP 4–8 per rat. A comparison of the stimulating effect of VP analogs is listed in Table 3 (Zhou et al., 1996). Furthermore, the peptide-enhancement was markedly depressed (47%) by 10 μg of ZDC(C)PR. In addition, by using a gel mobility shift assay and a synthetic probe encoding an AP1 binding fragment in the promoter region of NGF gene (from −18 to +48), the possible role of Fos expression in the peptide-induced process has been testified. These results clearly showed that NGF gene was one of the target genes responsible for memory-enhancing responses induced by VP 4–8 and the enhancement of NGF gene expression may be involved in the signaling pathway mediated by VP 4–8 receptor and *c-fos* gene expression.

Table 3
Relative expression of NGF mRNA in rat cerebral cortex and hippocampus[a]

Peptide	Cortex	Hippo-campus	Behavioral potency ED_{50} (μg/rat)
$(ZNCPR)_2$	1.4 ± o.3*	0.9 ± 0.4*	>10
ZDC(C)PR	1.5 ± 0.4*	0.9 ± 0.3*	>10
NLPR	3.1 ± 0.5**	1.9 ± 0.4**	0.05
Ac-YQNC-(C)PR-NH_2	4.1 ± 0.3**	2.2 ± 0.2**	0.08
VP 4–8	6.4 ± 0.5**	3.1 ± 0.8**	0.1
VP	1.6 ± 0.2**	0.9 ± 0.1*	0.48
OT	1.1 ± 0.1*	0.9 ± 0.2*	ND

[a] Northern blot data are represented as ratio (means ± SE) compared with saline group. *$P > 0.05$, **$P < 0.05$.

NGF plays important roles in the formation of memory through maintaining the septohippocampus cholinergic projections and can also improve the memory condition in diseases, such as Alzheimer's disease (AD) (Olson, 1993). Since VP 4–8 can enhance NGF expression in the rat brain, it is suggested that it could exert action on memory-impaired rats. In fact, studies on memory-impaired rats prepared by prenatal hypoxia showed that NGF expressions in the hippocampus and the cerebral cortex increased from a lower level to normal by the administration of NLPR, a tetrapeptide used as an agonist of VP 4–8 and the acquisition and maintenance of behavioral responses in these animals were improved concomitantly (Zhou et al., 1995b, 1996). These findings not only confirmed the role of NGF in the learning process, but also showed the involvement of VP 4–8 in this process.

Differential expression of neurotrophins

In addition to NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) are also widely distributed in the rat brain, especially abundant in hippocampus, but display distinct patterns of target specificity. For instance, BDNF and NGF but not NT3 are mainly targeted to the basal cholinergic neurons in adult rats (Phillips et al., 1990). The involvement of basal forebrain-septal cholinergic neurons in learning and memory has been well established (Araujo et al., 1990). The fields of termination of the septohippocampal cholinergic projections are maintained by a continuous trophic supply of NGF and BDNF from the target neurons, e.g., pyramidal and granular cells in the hippocampus (Ayer-LeLievre et al., 1988; Phillips et al., 1990). Therefore, continuous expression of these two neurotrophin genes in the limbic system is crucial in learning and memory processes (Gage et al., 1990).

Since VP 4–8 has effects on the long-term memory-enhancing process and on NGF gene expression, it is necessary to study its effects on the in vivo expression of BDNF and NT-3 genes in the same conditions. By using in situ hybridization and quantitative Northern blot analysis with cDNA and antisense RNA as probes, Zhou et al. (1997) found that, similar to NGF, BDNF expres-

sion was also significantly enhanced by VP 4–8 administration in rat cerebral cortex and hippocampus, but NT3 expression was not changed. VP showed a little inducing-effect on the expressions of NGF and BDNF, but the behaviorally active molecule oxytocin had no effect at all. These results indicated that NGF and BDNF genes were two of the target genes responsible for memory-enhancing responses.

In neurotrophins associated with the regulation of long-term memory, BDNF is thought to be another important member in addition to NGF (Ernfors et al., 1990; Phillips et al., 1990; Wetmore et al., 1990). Although BDNF might facilitate both short-term and long-term memory, the tremendous increase of BDNF mRNA transcription (4.1-fold in the rat cortex and 3.1-fold in the hippocampus) appearing 12 h after administration (s.c. 0.2 μg per rat) of VP 4–8 indicated the facilitation of the long-term process.

Although NT3 is abundant in the hippocampus, the role of this factor is at present not clear. It has been found that, in contrast to BDNF and NGF, neuronal expression of NT3 mRNA in the brain is not influenced either by kainic acid treatment or by kindled seizure (Ballarin et al., 1991; Ernfors et al., 1991). Interestingly, a similar differential effect was found in our studies: the expression of NT3 mRNA in rat hippocampus was not enhanced either by exogenous VP 4–8 or by VP. In view of the differential targeting of NGF, BDNF and NT3 in the area of basal forebrain cholinergic neurons (Phillips et al., 1990), a rational explanation for the differential inducing-effects of the peptide on neurotrophin gene expression is that the cells susceptible to VP 4–8 in the limbic system are those neurons which synthesize and secret neurotrophins, NGF and BDNF, to induce and maintain the cholinergic projections. In brief, our findings undoubtedly indicated the involvement of NGF and BDNF but not NT3 in the learning and memory processes by strengthening the basal cholinergic projection in adult rat brain.

Conclusions

From the data accumulated so far, some conclusions can be drawn: Firstly, as a neuropeptide VP 4–8 not only induces significant facilitation of passive avoidance behavior in a dose-dependent fashion but also facilitates remarkably acquisition and subsequent maintenance of brightness discrimination in immature and mature rat by neonatal administration. Secondly, studies on structure–activity relationships revealed that a relative compact structure is necessary for this peptide and its analogs to exert their behavioral activities. Thirdly, a new type of specific binding sites for VP 4–8, which differs from the known VP binding site has been found in many regions of the rat brain, especially in the limbic system. Fourthly, recent investigation in our laboratory is focused on the signal transduction in the rat brain induced by VP 4–8 and the results so far suggest a branching signaling pathway as depicted in Fig. 2.

Fig. 2 shows that VP 4–8 evoked a series of molecular events in the rat brain, including: binding specific receptor, stimulating IP_3 accumulation, potentiating synaptic transmission, inducing proto-oncogene *c-fos* transcription and stimulating neurotrophin genes expression, etc. Since memory can be described as a temporary and a long-lasting process, a memory-enhancer might affect neuronal action in two different ways: short-term (hours to days) and long-lasting (years). VP 4–8 produces a short-term memory enhancement by a rapid alteration of synaptic plasticity through activation of PKC and the phosphorylation of growth-associated protein

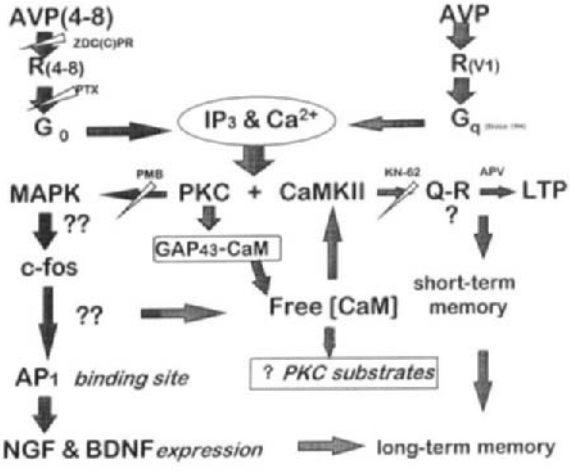

Fig. 2. A schematic signaling pathway of VP 4–8.

(GAP43) and CaMKII as well as a long-term effect by changing cellular structure via gene expression. As specific receptor binding is the trigger for intracellular signaling, further work on the nature of the receptor specific for VP 4–8 is being undertaken at the molecular level. Finally, our results not only suggest that the memory-enhancing peptide, VP 4–8 could be used as a probe to explore the molecular links coupled with the memory process, but also imply that VP 4–8 and related peptides may be potential candidates for treatment of memory disorders.

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CHAPTER 3.1.3

Vasopressin in the mammalian brain: The neurobiology of a mnemonic peptide

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We have sought to understand the mechanisms by which VP can enhance memory function and in the process determine whether VP fulfills the requirements for neurotransmitter status. The latter goal of proving the neurotransmitter status of VP has been achieved through our findings and the results of many of the scientists contributing to this volume. With respect to elucidating the mechanisms by which VP can enhance memory function, results of our work have shown that VP and its receptors are present in brain regions known to be involved in memory function, that release of VP is inhibited by a factor that inhibits memory function, that VP can significantly enhance the morphological complexity and outgrowth of neurons involved in memory function, that second messenger systems held to be involved in learning and memory, cyclic AMP and calcium signaling pathways, are potentiated and activated by VP, that electrophysiological models of memory function are induced by VP, and that when animals remember a learned association VP content in brain increases over time during the active phase of remembering. Collectively, these studies have taught us a great deal about the sites and mechanisms of VP action and have led us to pursue avenues of investigation that we would not have imagined 15 years ago when we began this work. We stand on the threshold of a new era in our research as we begin our studies of the role VP and its receptors play in the cerebral cortex. Thus far, results of these studies are quite exciting and promise to yield fascinating insights into the complexities of VP action in the most highly developed region of the mammalian brain, the cerebral cortex, the site of abstract reasoning, judgment, complex analysis and the repository of those memories that last a life-time.

Introduction

'When I don't take my vasopressin, I can't remember stuff sometimes and then I have to be reminded a lot of times. I get confused with a lot of stuff and then I can't think clearly and then it's very very hard to think. When I take my vasopressin I'm able to remember stuff much better, then I can think much more clearly and sometimes I even have a little fun.' Joseph Arminger, 9 year old boy with vasopressin (VP) deficiency.

Memory function, while a seemingly pervasive cognitive process, is also remarkably vulnerable to biological challenge. This characteristic was most evident in our early work studying individuals who had suffered very mild to severe brain damage. While these individuals varied dramatically in both the etiology of their brain damage and in the severity of the accompanying neuropsychological deficits, a problem that all of these individuals shared in common was a disturbing loss in memory function (Brinton, 1981). It was from this vantage point that we began our studies into the neurobiology of memory function. The decision to investigate the actions of VP as a molecule that could enlighten our knowledge of the neurobiology of memory had its foundation in the pioneering work of David De Wied and his colleagues. These investigators discovered that VP enhanced memory function in animals (De Wied, 1965, 1971, 1984; De Wied et al., 1984, 1988). Later analyses in non-human primates (Bartus et al., 1982) and in humans (Weingartner et al., 1981; Jolles, 1987) also found that VP enhanced memory function. The behavioral

data demonstrating that VP enhanced memory function was the catalyst for our, now, 15 year effort to determine the site(s) of VP action in brain and its mechanisms of action

When we began this work in the early 1980's, VP was not yet considered a neurotransmitter in brain. Our first efforts were, therefore, directed towards determining whether VP fulfilled the classical criteria of a neurotransmitter in brain (Bloom, 1990). To address this issue, we designed experiments that tested whether VP fulfilled the criteria of a neurotransmitter in the context of anatomical, physiological, genomic and behavioral mechanisms held to be biological substrates of memory. To conduct these investigations we have used an extensive armamentarium of experimental approaches that have included biochemical, physiological, cellular biological, electrophysiological, molecular biological, and behavioral strategies. We began our studies with an investigation of VP presence in brain and its release under conditions relevant to memory function. We then investigated the existence of VP receptors in the central nervous system, sites at which VP could regulate memory function. Following detection of VP receptor elements, we pursued the signal transduction system activated by those receptors. From there we have investigated the cellular, electrophysiological, and genomic actions induced by VP in brain. In addition, we have investigated whether VP content in brain changes during an active phase of remembering. The review that follows is focused on the work from our laboratory conducted over the past 10–15 years and is not meant to be an exhaustive review of the many excellent studies conducted over the years in other laboratories. However, in each of the sections, an attempt is made to evaluate our data with respect to the larger body of data in the field.

VP content and release in the mammalian brain

In the early 1980s there was still some debate regarding the status of VP as a neurotransmitter in the central nervous system. The first criteria required to establish a factor as a neurotransmitter was demonstration of its presence and release in brain (Bloom, 1990). VP immunoreactive fiber systems had been demonstrated in extrahypothalamic sites by Buijs, De Vries and coworkers (Buijs et al., 1983; deVries and Buijs, 1983; deVries et al., 1983). Based on these findings, we investigated whether detectable levels of the VP peptide were present in brain and in particular whether VP was present in brain regions known to be involved in learning and memory. To conduct this investigation, we developed a non-equilibrium radioimmunoassay that enhanced the level of detection of VP into the picomolar range (Brinton et al., 1983). We then applied this RIA to the measurement of VP in specific brain regions and found that VP was indeed present in the hypothalamic nuclei that synthesize VP, in the pituitary that stores it, and in target regions such as the hippocampus, amygdala, septum, thalamus substantia nigra, corpus striatum and thalamus (Brinton et al., 1983). These data demonstrated the presence of detectable levels of the VP peptide in brain and at sites involved in memory function. We then proceeded to investigate the release of VP in brain.

To explore whether VP was released under conditions relevant to memory function, we chose to analyze the effect of ethanol on VP content and release from brain because ethanol had been shown to impair memory function across a number of species including rodents, primates and humans (Brinton et al., 1986). We reasoned that if VP is a key regulator of memory function, then a substance such as ethanol should inhibit or impair the release of VP. It is well known that ethanol, at behaviorally relevant blood levels, induces a decrease in the plasma content of VP (Verney, 1947; Brinton et al., 1986). We therefore investigated the impact of behaviorally relevant blood ethanol concentrations on VP content in sites of VP synthesis and on select target regions. Acute in vivo administration of 1.6 g/kg of ethanol yielded a blood ethanol level of 126 mg% or 27 mM which is a blood ethanol concentration at which memory deficits are apparent. At this ethanol level, VP concentration rose, but not significantly, in the pituitary and the hypothalamic nuclei that synthesize VP, the paraventricular and supraoptic nuclei. The septum also showed an increase in VP content. Other brain regions examined, the amygdala, corpus striatum, hippocampus,

substantia nigra, and thalamus did not change in VP content relative to that of control animals. The increase in VP content in select brain regions was most likely due to dehydration and the concomitant increase in plasma osmolarity that occurs in response to 126 mg% blood ethanol concentration (Brinton et al., 1986). These data indicated that the decrease in VP content seen following acute ethanol exposure was not due to a decrease in VP content in either the sites that synthesize VP or in the site of storage and release into the periphery, the neurohypophysis. We then asked whether the same condition existed in animals chronically exposed to ethanol. Using a 10% ethanol liquid diet, which yielded a blood alcohol level of 88 ± 14 mg%, animals were chronically treated with ethanol for 2 weeks. No difference in VP content occurred in any of the brain regions examined. Together, the data demonstrated that acute ethanol exposure induced an increase in VP synthesis whereas chronic low levels of ethanol did not induce a change in VP content. These data indicated that the memory impairing effect of ethanol and the lower plasma levels of VP could not be explained by a decrease in available stores of VP and was therefore most likely due to an inhibition of release.

To explore the effect of ethanol on VP release, we used an in vitro slice preparation of the median eminence and examined the effect of varying concentrations of ethanol on VP release from the median eminence during potassium-induced depolarization. We selected the median eminence because it had a sufficiently large quantity of VP from which VP release could be reliably measured and was still within the brain proper (Brinton et al., 1983). Results of this experiment documented a biphasic inhibition of VP release following exposure to varying concentrations of ethanol. At behaviorally relevant concentrations of ethanol, ethanol induced a progressively greater and significant inhibition of depolarization-induced VP release. The ethanol-induced inhibition of VP release was apparent and significant at 5 mM ethanol and reached a maximal inhibitory effect at 25 mM ethanol (corresponding to a blood ethanol level of 115 mg%). It is noteworthy that at 115 mg% blood ethanol concentration, the concentration inducing maximal inhibition of VP release, cognitive and motor impair-ments are also apparent (Brinton et al., 1986). Ethanol concentrations that exceeded the LD_{50} of ethanol (> 60 mM ethanol or 300 mg% blood ethanol concentration) induced an increase in VP release which was most likely due to neuronal damage and not due to a physiological adaptive response.

These data indicated that ethanol concentrations sufficient to induce memory deficits also inhibited release of VP. The decrease in VP release can not be accounted for by a decrease in VP levels since acute in vivo treatment with ethanol that induced a similar blood ethanol concentration did not induce a decrease in VP content in any of the brain regions examined. In fact, acute ethanol treatment induced a modest but consistent rise in VP content in the hypothalamic nuclei that synthesize VP and in the posterior pituitary. In addition, chronic exposure to ethanol which induced a lower but still behaviorally relevant blood ethanol concentration did not change VP content in any of the brain regions examined. In contrast, in the in vitro preparation the same ethanol concentration achieved in the chronic ethanol study inhibited VP release. Considered together these data indicate that behaviorally relevant ethanol concentrations produced a 20–60% inhibition of VP release that was not due to a reduction in VP content. These data demonstrated for the first time that the reduction in VP plasma levels following ethanol exposure was not caused by ethanol-induced depletion of VP but was more likely due to an inhibition of release of available stores of VP. In addition, these data provided insight into the neurochemical basis of ethanol-induced memory dysfunction. Lastly, we were able to show that VP was released from neural tissue, thus fulfilling one of the criteria for neurotransmitter status, and that its release could be regulated by a factor that impairs memory function.

VP receptors in the mammalian brain

The anatomical distribution of neurotransmitter receptors provides a map that can illuminate or suggest the function a neurotransmitter serves in the brain. Towards that goal, we have, over the years, explored the distribution of VP receptors in the nervous system. Because of our interest in the

neurobiology of learning and memory, we were particularly curious as to whether VP receptors were expressed in brain regions critical to learning and memory. Results of our autoradiographic analyses in the rodent and postmortem human brain revealed that VP receptors are indeed present in numerous brain regions and are quite apparent in brain regions critical to learning and memory, such as the hippocampus and cerebral cortex (Brinton et al., 1984; Chen et al., 1993) (Fig. 1 and Tables 1 and 2).

VP receptors in the hippocampus

In the rodent hippocampus, we detected VP receptors in the pyramidal CA1, CA2, CA3 regions of Ammon's horn and the dentate gyrus (Brinton et al., 1984, 1991). Our observation of VP receptors in the hippocampus was consistent with the findings of Biegon et al. (1984), De Kloet et al. (1985) and Poulin et al. (1988).

A regional analysis revealed that the V_1 subtype of VP receptors were expressed in both the dorsal and ventral CA1, 2 and 3 regions. In the dorsal CA1, the V_1 VP receptor was fairly uniformly distributed from the rostral to caudal portions of CA1 whereas the distribution of V_1 VP receptors in the CA2 region sharply dropped in the caudal portion of CA2. V_1 VP receptors were expressed throughout the rostral and caudal extent of the CA3 region. Within the CA1 region, a detailed laminar analysis showed that the V_1 VP receptor was expressed in the strata oriens, radiatum and lacunosum-moleculare as well as in the pyramidal cell layer. The distribution of V_1 VP receptors within the dendritic zones of the hippocampus strongly suggested that V_1 VP receptors were present in zones of synaptic contact. Later studies, described below, showed that these receptors were postsynaptic in location.

In the region of the dentate gyrus, VP receptors were detected in both the dorsal and ventral portions. A rostral–caudal gradient of the receptors was evident throughout both the dorsal and ventral portions of the dentate gyrus. Within the granule cell layer a very low density of receptors was detected in the dorsal portion while a high density existed within the ventral portion which dropped off sharply as the layer proceeded caudally. Within the molecular layer, a remarkably high level of receptors were detected in the rostral portion which declined sharply in the more caudal portions. V_1 VP receptors were also apparent in the polymorph layer of the dentate gyrus. The rostral-caudal distribution of V_1 VP receptors within this region remained relatively high until the very last sections of the caudal portion.

The distribution of V_1 VP receptors within the hippocampus exhibited four important features. First, the abundance of the V_1 VP receptor was low relative to other neurotransmitter systems such as the beta adrenergic receptor, although in some lamina the abundance of the V_1 VP receptor was equal to that of the beta adrenergic receptor. Second, despite the low abundance, the V_1 VP receptor was consistently present in both the dorsal and ventral hippocampus. Third, the distribution of V_1 VP receptors exhibited a laminar specific expression. Fourth, within hippocampal subregions and within specific lamina, a rostral-caudal and dorsal-ventral gradient existed with the rostral and ventral regions generally exhibiting a greater abundance of V_1 VP receptor.

Investigation of the postmortem human brain for expression of VP receptors revealed a pattern of distribution that was analogous to what we had observed in the rodent hippocampus (see Fig. 1 and Table 1). VP receptors were detected in each of the CA subfields of Ammon's horn and within the dentate gyrus. In addition, VP receptors were detected in the subicular regions. Detection of VP receptors in the human hippocampus and cerebral cortex is an important finding when considering the generalizability of our findings in the rodent brain at both the receptor and effector levels of analysis to relevance for the human brain.

What is the functional significance of the expression of V_1 VP receptor in the hippocampus and within specific subfields of the hippocampus? To address this question one must return to the work of De Wied and his colleagues who have contributed most extensively to answering this question. The early work of Kovac and colleagues showed that injections of VP into either the dorsal or ventral

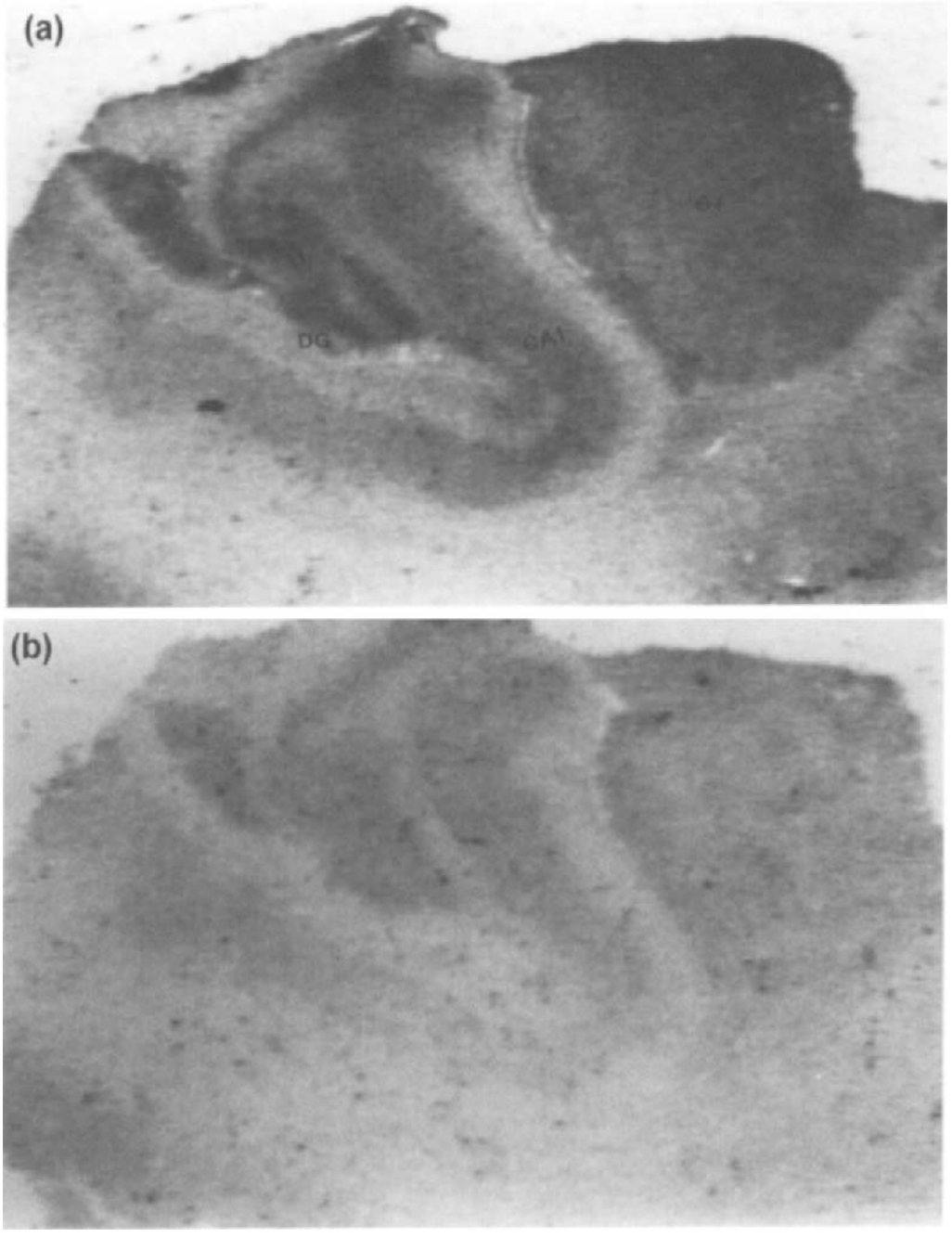

Fig. 1. Distribution of VP receptors in the hippocampus and cerebral cortex of the human postmortem brain. (A) Autoradiographic image of human brain section labeled with 10 nM [^{3}H]arginine vasopressin. VP receptors appear as dark and medium gray zones. (B) Section of postmortem human brain adjacent to that shown in A labeled with 10 nM [^{3}H]arginine vasopressin in the presence of 10 μM unlabeled arginine vasopressin. Specific binding as determined as residual binding in the presence of 10 μM unlabeled arginine vasopressin was 43%. CA, CA 1 region of hippocampus; DG, dentate gyrus; H, hilus; OT, occipitotemporal cortex. (From Duvernoy, *The Human Hippocampus*, 1988).

hippocampus significantly enhanced both consolidation and retrieval with injections of VP into the ventral hippocampus inducing the greatest response (Kovacs et al., 1986). These findings were later confirmed and extended in the mouse by Metzger and coworkers (Metzger et al., 1993). Our quantitative anatomical analysis of the V_1 VP receptors within the hippocampus fits the behavioral findings in that V_1 VP receptors were present in both the dorsal and ventral hippocampal lamina with the ventral hippocampal laminae expressing a greater abundance of receptors.

VP receptors in the cerebral cortex

More than a decade ago, our work resulted in the discovery of the VP receptor system in the cerebral cortex (Brinton et al., 1984). Results of these studies demonstrated recognition sites for VP throughout the cerebral cortex, from the frontal lobe to the occipital lobe. In addition, the recognition sites for VP appeared by autoradiography to be uniformly distributed throughout the cortical layers. Within the cerebral cortex, VP receptors were autoradiographically detected in each of the cortical layers using both radiolabeled VP (Brinton et al., 1984; see Fig. 1) and a radiolabeled specific V_{1a} receptor antagonist (Chen et al., 1993). Recent

Table 1
VP recognition sites in the human hippocampus[a]

Hippocampal region	[^{3}H]VP bound, fmol/mg protein mean ± SEM (*n*)
CA1 moleculare	25 ± 5.0 (4)
CA2 moleculare	22 ± 4.0 (4)
CA2 moleculare	34 ± 7.4 (4)
Hilus of dentate gyrus	30 ± 7.7 (4)
Dentate gyrus	29 ± 3.5 (4)
Granular layer of the dentate gyrus	19 ± 6.5 (4)
Subiculum moleculare	23 ± 4.6 (4)
Subiculum medial cellular band	18 ± 2.3 (4)
Subiculum extreme band	5 ± 1.6 (4)

[a] Optical density values were converted to fmol/mg protein values by using ^{3}H-labeled standards and a Drexel Unix-based microcomputer image analysis system.

Table 2
VP recognition sites in other brain regions of human brain[a]

Brain region	[^{3}H]VP bound, fmol/mg protein mean ± SEM (*n*)
Amygdala	23.6 ± 2.0 (4)
Frontal cortex	3.1 ± 2.2 (2)
Occipital cortex	4.3 ± 4.5 (2)
Occipito-temporal cortex	17.4 ± 0.7 (4)
Caudate	4.5 ± 3.8(3)
Lateral thalamus	5.4 ± 5.0 (4)
Medial thalamus	12.7 ± 9.3 (4)
Plexus blood vessels	35.8 ± 2.4 (4)

[a] Optical density values were converted to fmol/mg protein values by using ^{3}H-labeled standards and a Drexel Unix-based microcomputer image analysis system.

in situ hybridization localization of mRNA for the V_{1a} VP receptor in adult rat brain shows abundant receptor mRNA expression in cerebral cortex of adult male and female rats (Szot et al., 1994). The presence of mRNA for V_{1a} receptor in these brain regions supported our earlier autoradiographic findings that VP receptors do indeed exist in the cerebral cortex and that these receptors continue to be expressed in the adult cerebral cortex (Brinton et al., 1984; Chen et al., 1993).

Our recent work has investigated the expression of the mRNA for the V_{1a} receptor within the four cortical lobes and within 3 cortical cell types (Yamazaki et al., 1998). By using the reverse transcription polymerase chain reaction (RT-PCR) method to amplify a 350 base pair sequence corresponding to the coding region of the fifth through the seventh transmembrane domains of the V_{1a} receptor gene (Morel et al., 1992), expression of V_{1a} receptor mRNA was detected in cultured neurons, astroglia, and oligodendroglia derived from the entire cerebral cortex. The distribution of $V_{1a}R$ was further characterized by analyzing enriched cultures of neurons, astroglia, and oligodendroglia from each of four lobes (frontal, temporal, parietal and occipital) of the cerebral cortex. V_{1a} receptor mRNA expression was found in each of the cell types from each region of the cortex, suggesting that V_{1a} receptor is broadly

distributed throughout the developing rat cerebral cortex. The widespread expression of V_{1a} receptor mRNA coupled with its detection in three major cell types indicates that the V_{1a} VP receptor is broadly distributed throughout the cerebral cortex and is consistent with our earlier findings (Brinton et al., 1984; Chen et al., 1993).

The detection of V_{1a} receptors in the cerebral cortex was for many years a controversial finding because some investigators had observed a transient expression of V_1 receptors in the hippocampus and cerebral cortex (Patracca et al., 1986; Tribollet et al., 1991) whereas our work demonstrated the existence of VP receptors in the mature rat cerebral cortex (Brinton et al., 1984; Chen et al., 1993). The controversy was resolved when V_{1a} VP receptor mRNA in adult rat brain was detected by in situ hybridization in the cerebral cortex (Ostrowski et al., 1992) of adult male and female rats (Szot et al., 1994) and by amplification using the polymerase chain reaction technique (Lolait et al., 1995; Yamazaki et al., 1998).

Investigation of the postmortem human brain, a collaborative study conducted with Dr. Anat Biegon at the Weizmann Institute in Israel, revealed that VP receptors were also expressed within the human cortex (Brinton et al., 1988) (see Fig. 1 and Table 1). VP receptors were detected in frontal, occipital and occipito-temporal cortex (sections containing other cortical regions were not available). Based on our finding of VP receptors in the postmortem human cerebral cortex, our current work investigating the effector mechanisms associated with the VP receptor in cells derived from the rodent cortex will contribute to our understanding of the role the V_{1a} VP receptor plays in the function of the human cerebral cortex.

What is the functional significance of the expression of V_1 VP receptor in the cerebral cortex and in specific cell types within the cerebral cortex? To date, we know little that would answer that question directly. However, behavioral studies in rodents, from our own and other laboratories (Messing and Sparber, 1985; De Wied et al., 1988; Chambers et al., 1993), using a wide array of paradigms, have documented that VP can enhance long-term memory function, a well accepted function of the cerebral cortex. Messing and colleagues have found that the memory enhancing effects of VP are positively correlated with the complexity the task, the greater the task complexity, the greater the memory enhancement by VP (Messing and Sparber, 1985). In addition, behavioral studies from our laboratory have demonstrated that prolonged maintenance (weeks) of a one trial conditioned taste aversion is associated with significantly higher levels of VP in select brain regions (Chambers et al., 1993). Because long-term memory formation and behavioral tasks of increased complexity are associated with cortical function (Brinton, 1981; Goldman-Rakic, 1988), the behavioral effects of VP suggested to us that the VP receptors we detected in the cerebral cortex may be of functional importance and that elucidation of the cellular, biochemical and genomic events induced by VP in the cortex could lead to a greater understanding of the mechanisms underlying integration and long-term storage of information in the cerebral cortex. Specifically, our autoradiographic (Brinton et al., 1984; Chen et al., 1993) and molecular analyses (Yamazaki et al., 1998) of the $V1_a$ VP (VP) receptor in the cerebral cortex suggests that the dorsal-ventral distribution of $V1_a$ VP receptors could provide a micro-network for VP to influence integration of information within neural columnar processing units while the broad rostral-caudal distribution of V_{1a} receptors could provide a macro-network for VP to influence integration of information throughout the cortex.

We recently detected mRNA for the VP peptide in both the developing and mature cerebral cortex (Chen et al., 1997) which suggests the intriguing possibility that cells within the cerebral cortex could synthesize VP locally for local use within cortical circuits. Our current working hypothesis is that the V_{1a} VP receptor system, that is broadly distributed throughout the four main regions of the cerebral cortex and within the different cortical cell types, functions to mediate integration of information in the cerebral cortex.

VP receptors in other brain regions

While the foregoing discussion has focused on VP receptors in the hippocampus and cerebral cortex, results of our autoradiographic analysis

also revealed that VP receptors are present throughout the rodent brain: in the neuro and adenohypophysis, in the hypothalamic nuclei that synthesize VP, the paraventricular, supraoptic and suprachiasmatic nuclei, the median eminence, the lateral septum, the medial amygdala, the cerebellum and the dorsal and ventral horns of the spinal cord (Brinton et al., 1984). In the postmortem human brain, we detected VP receptors in the lateral and medial thalamus, the amygdala and the caudate in addition to the hippocampal regions and cerebral cortex (Brinton et al., 1988). The functional significance of VP receptors in many of these brain regions remains to be discovered.

VP and OT receptors in the developing telencephalon

Because our earlier work documented a neurotrophic effect of VP in the hippocampus (Brinton et al., 1994) and, more recently, in the cerebral cortex (Chen et al., 1996), these studies conducted with embryonic cultured neurons suggested to us that VP might play a role in embryonic brain development. To pursue this postulate, we investigated the developmental expression of VP and oxytocin (OT) receptor mRNA using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot hybridization (Chen et al., 1997). Results of these analyses documented that mRNAs for the V_{1a} and V_2 VP receptor subtypes were present in the telencephalon throughout the developmental time frame investigated, from embryonic day 12 to day 20. Expression levels for both VP receptor subtypes were increased on day 13 and differed slightly from embryonic day 13 to day 20. Messenger RNA for the VP peptide was also detected in the telencephalon throughout the developmental time frame investigated, from day 12 to day 20, and in the adult rat cerebral cortex indicating that VP could be synthesized within the rat cerebral cortex during rat embryonic development as well as in the adult cortex. OT receptor mRNA expression was present in the telencephalon from embryonic day 12 to day 20 as well, but expression levels for OT receptor mRNAs varied considerably from day 12 to day 20. No OT mRNA expression was detected during rat telencephalon development. Temporal patterns of VP receptor and VP peptide mRNA expression along with OT receptor mRNA suggest a temporal role for VP- and OT-mediated actions during rat telencephalon development.

Signal transduction systems activated by VP receptors

VP-induced neuromodulation

In this work, we were once again led by the findings of Kovacs et al. (1979) who showed that the memory enhancing effect of VP was lost following lesioning of the dorsal noradrenergic bundle, suggesting an interaction between norephinephrine (NE) and VP was required for the behavioral effects of VP. We pursued such a possibility by investigating the ability of VP to potentiate NE-induced cyclic AMP. Results of this study demonstrated that VP indeed potentiated NE-induced cyclic AMP formation in the hippocampus and that the potentiation was selective for beta adrenergic stimulated adenylate cyclase (Brinton and McEwen, 1989). Several other interesting features of VP-induced neuromodulation emerged from this investigation. First, in addition to the effect being dose-dependent and peptide specific, we also found that VP-induced neuromodulation was blocked by a V_1 receptor antagonist and by an antagonist to the calcium binding protein, calmodulin. We further found that VP-induced neuromodulation was a calcium dependent process. Surprisingly, the effect was dependent upon the concentration of extracellular calcium, suggesting that VP-induced neuromodulation was dependent upon an influx of extracellular calcium and that VP may even regulate calcium channels. We have pursued this hypothesis in our more recent studies and have indeed found that V_1 receptor activation leads to an influx of extracellular calcium (Brinton et al., 1994a; see below).

Our finding of VP-induced neuromodulation in the hippocampus led to two collaborative studies. The first study, conducted in collaboration with Dr. Gerard Boer at the Brain Research Institute in the Netherlands, investigated the impact of early postnatal VP treatment on the later expression of VP-induced neuromodulation. We initially hypothe-

sized that VP treatment would down regulate VP receptors, thereby abolishing the ability of VP to modulate adrenergic stimulated cyclase. Instead, VP neuromodulation was not lost. Surprisingly, NE-induced cyclic AMP accumulation was dramatically decreased (Fig. 2). VP still potentiated NE-induced cyclic AMP accumulation at the same magnitude as the controls. The total amount of cyclic AMP generated was decreased but the relative magnitude of the potentiation was the same. Thus, it appeared that VP had down-regulated the adrenergic response which suggested that a VP-NE receptor complex might exist.

Supporting evidence for a VP-NE receptor complex came from a totally different study. This investigation, conducted in collaboration with Dr. Ron DeKloet at the Rudolf Magnus Institute in the Netherlands, was designed to determine whether VP receptors were pre- or postsynaptic to adrenergic terminals. From the biochemical data alone we could not determine whether VP was acting to increase NE release which would result in an increase in NE-induced cyclic AMP accumulation or whether VP acted at a postsynaptic site to potentiate the biochemical process that leads to cyclic AMP production by adrenergic receptors. To address this question, lesions of the dorsal noradrenergic bundle were performed followed 10 days later by receptor autoradiography for VP receptors. This study also led to unexpected results. Autoradiographic evidence indicated that there was a site-specific upregulation of VP receptors following lesioning of the dorsal noradrenergic bundle (Table 3). These data answered the question that we initially addressed with respect to pre vs. postsynaptic localization. The continued presence of VP receptors following the dorsal noradrenergic bundle lesion indicated that some, if not all, of the VP receptors were postsynaptic to noradrenergic terminals. Secondly and surprisingly, we found that the loss of adrenergic input to the hippocampus resulted in an upregulation of the VP receptor system that modulates adrenergic stimulated cyclase. Taken together, results of these two studies

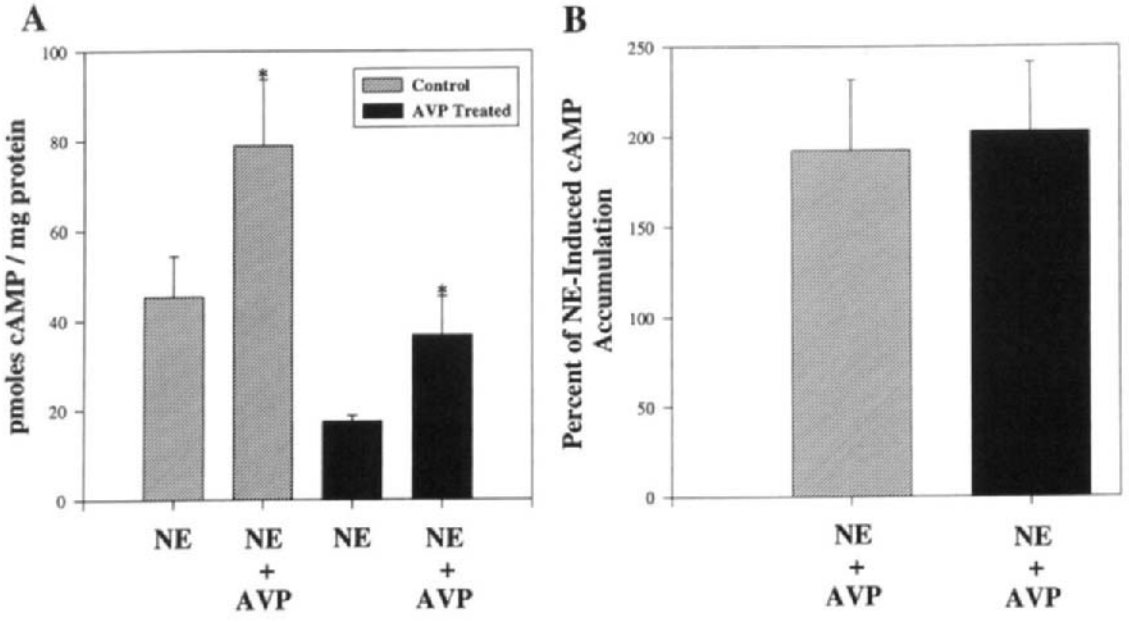

Fig. 2. Suggestive evidence for a VP-NE receptor complex. (A) Treatment of rat pups with 1 μg VP for 15 days following birth resulted in a pronounced diminution in cAMP formed in response to 10 μM NE in hippocampal slices. (B) However, no diminution in the magnitude of VP-induced potentiation occurred. These data support the hypothesis that at least a subset of NE and VP receptors exist as an interdependent complex such that high levels of VP can downregulate NE-induced cAMP formation without a loss in the ability of VP to act as a positive modulator.

suggest the intriguing possibility that a subpopulation of VP and β-adrenergic receptors exist as an adaptive and interactive complex. Thus, when an adaptive response occurs in one, a corresponding compensatory response occurs in the other.

Lastly, because our autoradiographic data had shown a widespread distribution of VP receptors in the hippocampus, we pursued the cellular specificity of VP-induced neuromodulation by selectively culturing neurons and glia from the hippocampus and investigated the ability of VP to potentiate noradrenergic-induced cyclic AMP accumulation in each cell type. Results of these studies showed that VP acted as a neuromodulator in hippocampal neurons and surprisingly, in glia (Brinton et al., 1991). These data indicate that in each of these cell types the complex interactive biochemical machinery necessary for the interaction between VP and NE exists. One of the benefits of the in vitro cell culture is that it provides an easily accessible developmental model system. Thus, we were able to investigate the development of VP-induced neuromodulation. The ability of VP to act as a neuromodulator was first evident in neurons after 3 days in culture (cultures were prepared from E18 rat embryo hippocampi). Prior to that time VP did not induce a neuromodulatory response. Hippocampal neurons seeded at a high density and grown for 3 days are highly differentiated nerve cells with elaborate and abundant cytoarchitecture. Thus, it is not until hippocampal neurons are highly differentiated do they exhibit the complex biochemical interaction manifested as neuromodulation.

The question that emerges from this work is: What purpose does neuromodulation serve? A simple, yet compelling hypothesis is that associative learning processes are dependent upon associative biochemical events (Brinton, 1990). In this light, associative and conditional neuromodulation may well serve as the biochemical analog for the processes that underlie associative learning which are behaviorally manifested as an enhancement of memory function. While the complexity and conditionality of neuromodulation greatly expands the signaling and information coding possibilities of neural networks, it is also a potential point of vulnerability. In this type of interactive system, there are many points at which the system can falter thereby resulting in a loss in memory function such as that observed in aging (Brinton et al., 1987).

VP-induced calcium signaling

Our investigations of VP-induced neuromodulation clearly indicated that a calcium dependent process was involved. It had been well-known for some time that activation of the V_1 receptor induces phosphatidylinositol hydrolysis at sites in the periphery and in the mature nervous system (Kirk and Michell, 1981; Bone et al., 1984; Stephens and Logan, 1986; Horn and Lightman, 1987; Shewey and Dorsa, 1988; Nathanson et al., 1992; Thibonnier, 1992). We therefore pursued VP activation of the phosphatidylinositol signaling pathway along with investigation of VP regulation of calcium uptake. To conduct our studies we took advantage of the cell culture technique that allowed us to

Table 3
Effect of 6-hydrozydopamine (6-OHDA) lesions on VP receptors in the rat hippocampus[a]

Structure	fmol [^{3}H]VP bound/mg protein		
	Control	6-OHDA lesion	% of control
Lateral septum	12.8 ± 1.05	12.9 ± 0.88	100
Pyramidal cell layer	11.7 ± 0.39	12.3 ± 0.72	105
Dorsal dentate gyrus	14.1 ± 0.82	14.7 ± 0.81	104
Ventral dentate gyrus	14.5 ± 1.03	16.6 ± 0.33*	111
Amygdalo-hippocampal area	14.6 ± 0.72	17.9 ± 0.23 **	123

[a] Values represent means ± SEM; $n = 6$/condition. Data were analyzed by paired Student's t-test, $^*P < 0.02$, $^{**}P < 0.003$.

selectively culture neurons, astrocytes and oligodendrocytes. By using the selective culture paradigm, we could investigate the expression of the VP receptor and the signal transduction pathway activated by VP in a specific cell type. In addition, we could study the developmental expression of both the receptor and its effector mechanism and ultimately investigate the cellular function of VP in each cell type.

Investigation of the effector mechanism of the VP receptor in cultured hippocampal neurons revealed that V_1 VP receptor activation leads to activation of the phosphatidylinositol signaling pathway, uptake of calcium from the extracellular medium and induction of complex intracellular calcium signals (Brinton et al., 1994). In cultured hippocampal neurons prelabeled with [^{3}H]myo-inositol, VP induced a significant accumulation of [^{3}H]inositol-1-phosphate ([^{3}H]IP_1). The selective V_1 VP receptor agonist, [Phe^2,Orn^2]-vasotocin, induced a significant accumulation of [^{3}H]IP_1 whereas a selective V_2 VP receptor agonist, [deamino^1, D-Arg^8]-vasopressin, did not. Moreover, V_1 agonist-induced accumulation of [^{3}H]IP_1 was blocked by the selective V_1 VP receptor antagonist d(CH_2)$_5$ [Tyr(Me)^2]-VP. V_1 agonist-induced accumulation of [^{3}H]IP_1 was concentration dependent and exhibited a steep inverted U-shaped curve that included both stimulation and inhibition of [^{3}H]IP_1 accumulation. Time course analysis of V_1 agonist-induced accumulation of [^{3}H]IP_1revealed significant increase by 20 min which continued to be significantly elevated for 60 min. Investigation of the effect of closely related peptides on [^{3}H]IP_1 accumulation indicated that the VP metabolite peptide VP 4–9 and OT significantly increased [^{3}H]IP_1 accumulation whereas the VP metabolite peptide VP 4–8 did not. VP 4–9 and OT induced [^{3}H]IP_1accumulation were blocked by the V_1 VP receptor antagonist d(CH_2)$_5$ [Tyr(Me)^2]-VP.

V_1 receptor activation in cultured hippocampal neurons was associated with a pronounced rise in intracellular calcium (Brinton et al., 1994a). Results of calcium fluorometry studies indicated that V_1 agonist exposure induced a marked and sustained rise in intracellular calcium that exhibited oscillations. Interestingly, absence of calcium in the extracellular medium abolished both the rise in intracellular calcium and the appearance of oscillations. The loss of the intracellular calcium signal is not due to a failure to induce PIP_2 hydrolysis since activation of the phosphatidylinositol pathway occurred in the absence of extracellular calcium. Since PIP_2 hydrolysis could be induced in the absence of extracellular calcium but the rise in intracellular calcium could not, it suggested that the rise in intracellular calcium required an influx of extracellular calcium.

We therefore investigated whether V_1 receptor activation would induce an uptake of calcium from the extracellular compartment. Results of these studies demonstrated that exposure to V_1 agonist (250 nM) induced a highly significant increase in $^{45}Ca^{2+}$ uptake from the extracellular medium within 5 s of exposure which remained significantly greater than basal for 300 s. The increase in $^{45}Ca^{2+}$uptake was followed by a significant inhibition of uptake by 20 min of exposure. Taken together, these data document that V_1 receptor activation in hippocampal neurons leads to activation of two calcium signaling pathways, the phosphatidylinositol second messenger system and uptake of extracellular calcium. We are intrigued by VP-induced calcium uptake and are currently pursuing VP activation of calcium channels.

Earlier autoradiographic studies from our laboratory detected VP receptors in cortical regions, and more recently, we have detected mRNA for the V_{1a} VP receptors (V_{1a}Rs) in cortical neurons. To determine whether these recognition sites were functional receptors, we pursued the signal transduction mechanism associated with the V_{1a} VP receptor in enriched cultures of cortical neurons (Son and Brinton, 1998). Results of these studies demonstrated that exposure of cortical neurons to the selective V_1 VP receptor agonist, [Phe^2,Orn^8]-vasotocin, (V_1 agonist) induced a significant accumulation of [^{3}H]IP_1. V_1 agonist-induced accumulation of [^{3}H]IP_1 was concentration dependent and exhibited a linear dose response curve. Time course analysis of V_1 agonist-induced accumulation of [^{3}H]IP_1 revealed a significant increase by 20 min which then decreased gradually over the remaining 60 min observation period. Investigation of the effect of closely related peptides on [^{3}H]IP_1 accumulation indicated that all of the VP metabolites

and well as OT significantly increased $[^3H]IP_1$ accumulation. Interestingly, all of the VP metabolites contained a common tripeptide sequence (Asn-Cys-Pro) which we hypothesize is the amino acid sequence required for receptor activation. Results of calcium fluorometry studies indicated that V_1 agonist exposure induced a marked and sustained rise in intracellular calcium which was abolished in the absence of extracellular calcium. The loss of the rise in intracellular calcium was not due to a failure to induce PIP_2 hydrolysis since activation of the phosphatidylinositol pathway occurred in the absence of extracellular calcium. V_1 agonist activation of calcium influx was then investigated. V_1 agonist-induced $^{45}Ca^{2+}$ uptake was concentration dependent with a biphasic time course at 250 nM. To determine the VP subtype specificity, we investigated the ability of a selective $V_{1a}R$ to block both the induction of IP and calcium uptake. Results of these studies demonstrated that the selective $V_{1a}R$ antagonist blocked both of these biochemical signaling pathways. Pre-incubation with the L-type calcium channel blocker, nifedipine, blocked V_1 agonist-induced calcium influx suggesting V_1 agonist-induced L-type calcium channel activation in cortical neurons. Furthermore, V_1 agonist-induced calcium influx was blocked by both bisindolyleimide I (PKC inhibitor) and U-73122 (PLC inhibitor) suggesting a modulation of V_1 agonist-induced L-type calcium channel activation by downstream components of the phosphatidylinositol signaling pathway such as protein kinase C. These results indicated that in cultured cortical neurons, V_{1a} VP receptor activation led to induction of the phosphatidylinositol signaling pathway, influx of extracellular calcium via L-type calcium channel activation, and a rise in intracellular calcium which was dependent on V_{1a} receptor activated influx of extracellular calcium (Son and Brinton, 1998). Finally, we also found that VP induced calcium signaling in cultured cortical astrocytes and oligodendrocytes (Son and Brinton, 1996, 1997). These data are the first to demonstrate an effector mechanism for the V_1 VP receptor in the cerebral cortex which may contribute to the ability of VP to enhance long-term memory function.

While our work has extensively utilized the advantages of the cell culture preparation, the question arises as to whether findings derived from embryonic cells in culture are applicable to the mature brain. Such a wide gap in milieus might, at first glance, may seem far to broad to allow any generalization from the in vitro culture condition to the in vivo situation. However, when one considers the mechanisms involved, there is increasing recognition of the parallels that exist between the biochemical and genomic pathways mediating learning and memory and those mediating cellular development (Jessell and Kandel, 1993). A rise in intracellular calcium, induced by stimulating the phosphoinositide signaling pathway or by activation of calcium channels, is a critical and pivotal event in regulating both cellular growth (reviewed in Berridge, 1993) and the mechanisms associated with learning and memory (Lynch et al., 1983; Malenka et al., 1988, 1992; Xie et al., 1992). Thus, we are reasonably confident that results derived from the cell culture preparation are indeed applicable to the mature brain and moreover, can provide important information relevant to understanding the signal transduction systems activated by VP in brain regions involved in learning and memory.

VP-induction of cyclic AMP formation in neural cells

In the process of searching for a homogenous neuron population that we could use to investigate VP-induced neuromodulation, we came upon an interesting finding in neuroblastoma cells. We found that VP induced cyclic AMP formation in the human cholinergic neuroblastoma cell line MC XI C (Davies et al., 1986). Of equal interest was the finding, that while these cells expressed β-adrenergic receptors that when activated induced cyclic AMP formation, the neuromodulatory effect of VP was not evident. We do not yet know whether these cells express the V_{1a} VP receptor, however, these cells could be an excellent model system to further investigate the development of VP-induced neuromodulation. We questioned whether VP-induction of cyclic AMP might be specific to neuroblastoma cells. This possibility was eliminated when we discovered that VP also induced cyclic AMP formation in cultured hippocampal neurons (Brinton and Brownson, 1993). VP-induction of

cyclic AMP was modestly apparent by the second day in culture, reached significant levels by the third day and was maximal on the fourth day of culture. By the fifth day in culture, VP no longer induced a significant increase in cyclic AMP. Together, these data demonstrate first that a V_2 like receptor, which we categorized as the V_{2b} receptor, can be expressed in neural tissue. Secondly, they document a developmental expression of a V_{2b} receptor that is expressed as early as the neuroblast stage of development and that continues to be expressed until late in embryonic neural development. Indeed, our analysis of V_2 receptor gene expression supports the biochemical data. We have found V_2 receptor gene expression in the brains of embryos as early as embryonic day 14 (Chen et al., unpublished observation). While the functional role of the V_2 receptor in brain remains to be determined, its developmental expression suggests the intriguing possibility that the V_{2b} receptor may play a role in the embryonic phase of nervous system development.

VP-induced neurotrophism

We pursued the functional significance of VP activation of second messenger systems by investigating the impact of VP on the outgrowth of neuronal processes. We selected this measure of cellular function because of the role neuronal morphology plays in the processes of learning and memory. It has been hypothesized that growth in the projections and synaptic connections of nerve cells is one of the fundamental mechanisms involved in memory formation (Ramon y Cajal, 1911; Wenzel et al., 1980; Vrensen, 1981; Black et al., 1990; Brinton, 1991). Thus, if learning and memory are associated with the enhancement of neuronal morphological features, a factor that enhances memory should also enhance the morphological complexity of nerve cells.

We initially tested this hypothesis using neurons derived from the cephalic portion of the *Xenopus* developing nervous system (Brinton and Gruener, 1987). Results of this study showed that in these neurons, VP induced a highly significant increase in neuronal process outgrowth. The increase in neuronal morphological complexity was evident within 12 h of VP exposure and was maximal by 24 h. Results of this study encouraged us to further pursue the neurotrophic action of VP in neurons derived from the mammalian hippocampus and cerebral cortex.

Our first series of studies using mammalian neurons investigated the neurotrophic action of VP in cultured hippocampal neurons (Brinton et al., 1994b). Results of these studies demonstrated that in the presence of serum, VP (1 μM), induced a significant increase in the number of neurites, in neuritic length and in neurite diameter following 48 h of exposure. Morphological complexity was also enhanced as indicated by a significant increase in the number of filopodia/branches, in the sum of branch lengths and in the number of branch bifurcation points. The number of microspikes decorating neuritic branches was also significantly increased following VP exposure.

To determine whether the neurotrophic effect of VP was dependent upon factors present in serum, hippocampal nerve cells were cultured in serum free media and exposed to 100–1000 nM VP. Results of these studies demonstrated that in a serum free media, VP induced significant enhancement of hippocampal nerve cell outgrowth and that the minimally effective concentration was reduced from 1 μM, as required in the presence serum, to 100 nM in the absence of serum. In addition, the time required for a significant increase in nerve cell growth decreased from 48 to 24 h. These results demonstrate that VP-induced neurotrophism is a direct effect and not dependent upon unidentified factors in serum.

Even more rapid effects of VP on hippocampal nerve cell growth could be observed when hippocampal neurons were exposed to high concentrations of VP (10 μM). Exposure to VP under these conditions resulted in a significant increase in the micro features of hippocampal morphology within just 10 min of exposure.

VP-induced neurotrophism was found to be mediated by V_1 receptor activation. Significant enhancement of nerve cell growth occurred following exposure to V_1 receptor agonist (100–1000 nM) whereas exposure to V_2 receptor agonist (100–1000 nM) did not increase any of the morphological parameters measured.

If VP-induced neurotrophism is indeed related to enhanced memory function and if neuronal outgrowth is a marker for information storage, then VP should enhance the morphological complexity of neurons derived from a brain region long held to be the site of long-term memory, the cerebral cortex. We therefore have begun an investigation of the neurotrophic action of VP in the cerebral cortex. Initial results of these studies have demonstrated that exposure to V_1 agonist significantly increased the growth of cortical neurons as indicated by a significant increase in the length of neurites ($P < 0.01$), the number of branches ($P < 0.01$), branch length ($P < 0.01$), the number of branch bifurcation points ($P < 0.01$) and in the number of microspikes ($P < 0.01$). The dose response profile of V_1 agonist-induced neurotrophism exhibited an inverted U-shaped function with 100 nM inducing the greatest increase in cortical nerve cell growth. The inverted-U shaped dose response is consistent with all V_1 agonist responses that we have previously observed. Our current work is investigating the regional selectivity of VP-induced neurotrophism in cortical neurons derived from the four lobes of the cerebral cortex. In addition, we are pursuing the biochemical and genomic mechanisms that mediate VP-induced neurotrophism.

In summary, results of our investigations using neurons derived from *Xenopus*, the hippocampus and the cerebral cortex document that VP can exert a significant neurotrophic effect. In addition, we have found that the neurotrophic effect is mediated by the V_1 type of VP receptor. Importantly, the neurotrophic action of VP was found to be a direct effect and not dependent upon factors present in serum. Collectively, these data document that VP, which enhances memory function, significantly increased the morphological features of neurons derived from brain regions involved in memory function. The ability of VP to significantly increase the cytoarchitecture of neurons derived from the hippocampus and cerebral cortex is consistent with the structural enhancement of nerve cell morphology that has been postulated to be an integral step in the cellular process leading to information storage in the nervous system. A growing body of data showing that neurotransmitters can act both as mediators of synaptic transmission and as neurotrophic agents during development and in the mature nervous system has led to the acceptance of neurotransmitters as pluripotential agents. As such, a neurotransmitter can act instructively as a chemical messenger for information transfer and/or as a neurotrophic agent depending on the responsive cell population (reviewed in Mattson, 1988; Patterson and Nawa, 1993). Moreover, these functions need not be mutually exclusive. It is entirely possible that a neurotransmitter can act both as an agent of information transfer between cells and as a trophic agent at the same site. Results of studies of VP action in brain, reviewed here and else where in other chapters, strongly suggest that this neural peptide falls within the category of neurotransmitter factors that can both influence synaptic transmission and act as a neurotrophic agent. Together, these studies impact our understanding of VP action in brain, particularly during neuronal development, and may be relevant to discerning the morphological correlates of VP enhancement of memory function in the mature brain.

VP-induced long-lasting potentiation of synaptic transmission

VP receptors are present in both the developing and mature dentate gyrus of the rat brain and are of the V_1 subtype. Because VP has been shown to influence memory function when injected into the dentate gyrus (Kovacs et al., 1979), the influence of this peptide on an electrophysiological model of learning and memory in the hippocampal slice preparation was investigated. Results of these studies showed that nanomolar concentrations of VP induced a prolonged increase in the amplitude and slope of the evoked population response in the presence of 1.5 mM calcium (Chen et al., 1993). Moreover, the expression of the VP-induced potentiation of the excitatory postsynaptic potential (EPSP) persisted following removal of VP from the perfusion medium. To distinguish VP-induced potentiation from that induced by glutamate, we termed this potentiation long-term VP potentiation or LTVP. Peptide specificity analysis documented that the closely related peptide, OT had no effect. In contrast, a selective V_1 antagonist (the same one

used in the calcium signaling and neurotrophic studies) blocked the induction of LTVP. Once again we observed that extracellular calcium played a decisive regulatory role in the expression of VP-induced responses. In the presence of 1.5 mM calcium VP induced a robust and long lasting potentiation of the EPSP. In striking contrast, in the presence of 2.5 mM calcium, VP induced a pronounced and long lasting depression of the EPSP. Together, these data indicate that VP exerts long lasting effects upon synaptic transmission in the dentate gyrus. In the presence of physiologically relevant concentrations of calcium, VP induced a long lasting enhancement of synaptic transmission. In the presence of supraphysiological levels of calcium, VP decreased synaptic transmission. This latter finding suggests that VP could actually exert a protective effect on neurons in the presence of pathological levels of extracellular calcium. VP induction of LTVP provides two supporting lines of evidence. First, VP potentiates synaptic transmission in a brain region critical to the processing and encoding of information, the dentate gyrus. Second, VP induction of LTVP provides support for the hypothesis that LTP is an electrophysiological model of memory (Berger and Bassett, 1992). An important study relevant to our finding of VP-induced LTVP is that of van den Hoof et al. (1989). These investigators found that VP was able to maintain long-term potentiation in slices of rat lateral septum. Together, results of these two studies indicate that VP can both induce and maintain enhancement of synaptic transmission.

VP-induced glial responses

When we detected mRNA for the V_{1a} receptor in cultured astrocytes (Yamazaki et al., 1998) it came as no surprise since our earlier autoradiographic data indicated a wide spread distribution of V_1 receptors suggesting to us that at least a portion of the autoradiographic signal might be from glial cells. We went on to show that these receptors were consistent with a V_1 type of receptor and activate phosphatidylinositol hydrolysis (Brinton and Gonzalez, 1993; Son and Brinton, 1997). One question that remains to be answered is the functional significance of the V_1 receptor in astrocytes in the cerebral cortex (Yamazaki et al., 1997a,b). To date we have no conclusive answer but we are actively pursuing the down stream events that are induced by V_{1a} receptor activation.

VP-induced gene expression

We investigated VP regulation of gene expression in the hippocampus for three reasons. First, it has been known for quite some time that long-term memory is dependent upon protein synthesis (Ramon y Cajal, 1911; Flexner and Flexner, 1966; Goelet et al., 1986). Since VP had been shown to enhance long-term memory (De Wied et al., 1988), it was reasonable to hypothesize that VP would regulate gene expression. Second, we had shown that VP could significantly increase hippocampal nerve cell growth (Brinton et al., 1994b) suggesting the possibility of de novo protein synthesis. Third, we had shown that VP induced calcium signaling in hippocampal neurons. Calcium is well known to regulate gene expression (Berridge, 1993). To pursue this investigation, we again utilized the cell culture preparation although in this instance we used a mixed culture preparation in which neurons and glia were present. We initially used the mixed culture preparation in order to assess gene expression in both neurons and glia. Genes selected for investigation were immediate early response genes that had been found to be induced during learning and memory or by factors that regulate learning and memory (Bartel et al., 1989; Sheng and Greenberg, 1990; Worley et al., 1990; Morgan and Curran, 1991).

Results of this investigation were surprising. VP did not induce expression of several immediate early response genes such as c-fos, c-jun or c-myc in cultured hippocampal neurons. Instead, VP induced a highly significant increase in the expression of the immediate early response gene, NGFI-A (also known as *zif/268*, ZENK, egr-1, krox 24) (Brinton et al., 1998). The NGFI-A gene encodes a transcription factor containing three zinc fingers proposed to regulate the expression of other genes over a longer period of time (Christy et al., 1988; Sukhatme et al., 1988; Pavletich and Pabo, 1991; Sukhatme, 1992). Cultured

hippocampal glial cells were exposed to VP or a selective V_1 VP receptor agonist and in situ hybridization conducted to detect NGFI-A mRNA. Results of these experiments demonstrated that VP-induced a highly significant dose dependent increase in the number of cells expressing NGFI-A. Studies to determine the receptor subtype mediating VP induction of NGFI-A were conducted utilizing the specific V_1 agonist, [Phe^2, Orn^8]-vasotocin. The V_1 receptor agonist induced a highly significant dose dependent increase in the number of grains per NGFI-A positive cell. Time course analysis demonstrated that V_1 agonist induction of NGFI-A occurred within 5 min, was maximally induced at 15 min of exposure and exhibited a gradual decline within 30 min of exposure which continued to decline over a 60 min time course. Glial cell responsivity was selective in that VP and V_1 agonist induction of NGFI-A occurred in a subpopulation of glial cells. Within a sea of glial cells, VP and the V_1 receptor agonist would induce islands of NGFI-A positive cells. Results of combined immunocytochemical labeling for the astrocyte specific marker, GFAP, and in situ hybridization for NGFI-A mRNA demonstrated that V_1 agonist-induced NGFI-A mRNA expression occurred in GFAP positive cells. We observed no evidence for V_1 agonist induction of NGFI-A in neurons. Collectively, these data document that VP, acting via V_1 VP receptors, induces a highly significant increase in NGFI-A expression in select GFAP positive hippocampal astrocytes.

The ability of VP, a memory enhancing neuropeptide, to induce NGFI-A gene expression, a gene that has been linked to processes involved in learning and memory (Wisden et al., 1990; Wallace et al., 1995), in cultured hippocampal glial cells raises several interesting and perhaps overlapping functional possibilities. Glial cells have long been appreciated for the supportive role they play in maintaining nerve cell viability and integrity of neuronal circuitry (Gless, 1955; Galambos, 1961; Laming, 1989; Steward et al., 1991). It is intriguing to speculate that VP induction of NGFI-A in glial cells may provide a supportive role in maintaining the viability and integrity of neuronal circuitry induced by the neurotrophic effect of VP in hippocampal neurons (Brinton et al., 1994). While learning and memory are typically considered the functional domain of neurons, increasing evidence suggests a critical role of glia in cognitive function (Laming, 1989). Such a postulate has not been traditionally well received; nevertheless, researchers have suggested such a possibility since 1886 (Galambos, 1961). Recently, the work of Greenough and colleagues has demonstrated that astrocytes respond to learning-induced increases in synaptic number and/or activity in a two phase manner. First, glial cells increase in cell surface during the process of learning (Anderson et al., 1994). Following the initial hypertropic response, Greenough and colleagues then observed glial cell proliferation (Anderson et al., 1994). These data demonstrate that astrocytes undergo changes in both cell size and cell number as part of the dynamic restructuring of neural circuits associated with learning and memory. Another intriguing finding from the Greenough group is the correspondence between sites of NGFI-A induction by exposure to complex environments and the sites of morphological plasticity. The hippocampus was one of the regions that showed NGFI-A induction and which exhibited morphological changes during and following learning and memory (Wallace et al., 1995). The fact that VP is a potent mitogen in other systems and that VP induces NGFI-A gene expression which is associated with mitogenesis in other systems, provides a promising foundation on which to pursue VP-induced mitogenesis in hippocampal glial cells.

VP and conditioned taste aversion

We undertook a study of the role VP might play in learning the conditioned taste aversion (CTA) association and/or in maintaining the CTA because the acquisition of a CTA in male rats is robustly learned within just one learning trial and can be maintained for extended periods of time from weeks to months (Garcia et al., 1955). This particular behavior was especially appealing to us because the association can be learned within just one trial, similar to many associations that humans make, and because the association can be maintained for long periods of time, again paralleling what is observed in the

human. For these reasons we viewed CTA as an excellent model paradigm to investigate the role VP might play in learning the association and/or in maintaining the association.

We were not alone in our choice of the CTA paradigm to investigate the role of VP in maintenance of learned association. Cooper et al. (1980) found that systemic administration of VP increased the time animals maintain the CTA while Brot et al. (1989) found that chronic central administration of an VP antagonist significantly decreased the time to extinguish a CTA. Our study did not administer exogenous VP to the animals but rather asked what is the in vivo VP response during the different phases of acquiring and maintaining the CTA.

The state of fluid hydration is one factor that strongly modulates extinction times in rats. The rate of extinction of a CTA is markedly faster in fluid deprived rats compared to non fluid deprived rats (Chambers and Sengstake, 1978). VP is well known as a primary hormone in fluid homeostasis (Dorsa and Bottemiller, 1983) and thus it seemed reasonable to postulate that changes in VP brain content in fluid deprived animals might underlie the faster extinction rate in these animals. In the fluid deprivation paradigm, animals are fluid deprived for 23 h each day with exposure to water for 1 h per day. The behavioral tests are of the two-bottle type (i.e. the animals are always given water in addition to the test solution during the 1 h daily test period), therefore the fast extinction in fluid deprived males is not a forced extinction due to thirst. This is the procedure we used for our experiment in which we investigated whether endogenous changes in VP content occurred in select brain regions during the extinction of a CTA in male rats not deprived of water, who can maintain the CTA for weeks to months, compared to those deprived of water for 23 h with 1 h of water access per day who extinguish the CTA much more rapidly.

VP levels were measured in known and putative brain regions involved in the neural circuitry of a CTA as well as in two control brain regions, the medial septum and insular cortex. Known and putative brain regions included the nucleus tractus solitarius (NTS) (Kiefer, 1985), lateral septum (LS), medial amygdala (AMe), bed nucleus of the stria terminalis (BNST) (Yamamoto et al., 1981), gustatory/visceral insular cortex (IC) (Yamamoto et al., 1981). The hypothalamic paraventricular nucleus (PVN) was selected because it is a primary source of VP and has reciprocal connections with the nucleus tractus solitarius (NTS) (Jean, 1991; Raggenbass et al., 1989). VP content was determined by radioimmunoassay as described in Brinton et al. (1983) in the seven brain regions. VP levels were determined at three different behavioral time points: following 23 h of fluid deprivation (Pre-CTA), following 10 days of fluid deprivation + acquisition of the CTA + one day of extinction trial (during which animals demonstrated a strong aversion; Trial 1) and following extinction of the CTA (Ext Day) in the fluid deprived animals at which time none of the non-deprived animals had extinguished the CTA.

Results of this experiment were surprising. We had anticipated that the fluid deprived rats that rapidly extinguished the CTA would exhibit low VP content in brain. While that was relatively true, what was surprising is that the real change occurred in the control animals that maintained the CTA. Control animals that had maintained the CTA exhibited higher VP levels whereas the fluid deprived animals showed no rise in their VP content following the acquisition of the CTA.

Significantly different patterns of VP content were exhibited in two sites of VP synthesis, the PVN and the AMe (see Figs. 3 and 4). In the PVN of fluid deprived animals, VP content rose significantly following 23 h of fluid deprivation and then declined over time. A significant reduction in VP content in the PVN of deprived animals occurred by Trial 1 (the first day of extinction testing, and on the day that they extinguished the CTA, Ext Day). In striking contrast, the non-deprived animals exhibited a significant increase in the VP content of the PVN at the Trial 1 and on the day when their yoked deprived counterpart had extinguished the CTA. The significant increase in the VP content of the PVN of non-deprived animals occurred during maintenance of the CTA. None of the control animals had extinguished the CTA by the time the fluid deprived animals had extinguished the CTA (see Table 2).

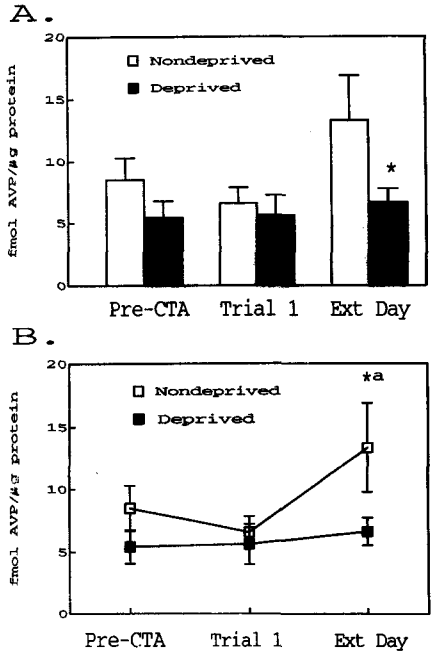

Fig. 3. Changes in VP content in the paraventricular nucleus (PVN) between deprived and non-deprived animals (A) and within treatment groups across behavioral time points (B). Data are presented as mean (±SEM) pmol VP/μg protein in the PVN of non-deprived and fluid deprived Fischer 344 male rats after a 23 h period before any conditioned taste aversion tests (Pre-CTA), after the first extinction trial (Trial 1), and at the day of extinction for deprived animals (Ext Day) but when the non-deprived animals continued to maintain the CTA ($N = 66$). (A) A significant difference between deprived ($n = 9$) and non-deprived animals ($n = 12$) occurred at the Pre-CTA behavioral time point (***$P <$ 0.0000, significantly different from non-deprived Pre-CTA, at the Trial 1 behavioral time point (*$P < 0.05$, significantly different from non-deprived Trial 1; deprived $n =$ 12, non-deprived $n = 10$) and at the day of extinction for deprived animals (***$P < 0.0003$, significantly different from non-deprived Ext Day; deprived $n = 12$, non-deprived $n = 11$). (B) A significant increase in VP content of the PVN occurred over time in the non-deprived group which was evident by the Trial 1 behavioral time point ([a]significantly different from non-deprived Pre-CTA, *$P < 0.03$) and at the final day of extinction testing ([b]significantly different from non-deprived Pre-CTA, **$P < 0.007$). In contrast, a significant decrease in VP content in the PVN of fluid deprived animals occurred at the Trial 1 behavioral time point and on the day of extinction ([c]significantly different from deprived Pre-CTA, ***$P < 0.0000$).

The medial amygdala also exhibited distinct patterns of VP content between the deprived and non-deprived animals. At the time when the control non-deprived animals were maintaining the CTA, VP content in the AMe significantly increased whereas when the deprived animals had extinguished the CTA (Ext Day) they exhibited significantly lower VP content in the AMe. Given that the amygdala and its principle VPergic target site, the hippocampus, are brain structures long known to be involved in memory processes, it is reasonable to suggest that these two brain structures could be an obligatory component to maintenance of a learned association such as occurs in the CTA paradigm. This postulate is supported by our findings that VP induces long-term potentiation in the dentate gyrus (Chen et al., 1993) and by our findings that VP enhances hippocampal nerve cell growth (Brinton et al., 1994). Both long-term potentiation and increased neuronal process outgrowth are cellular mechanisms postulated to be involved in memory function (reviewed in Brinton, 1991; Berger and Bassett, 1992; Jessell and Kandel, 1993).

In the BNST, the third site of VP synthesis, VP content increased significantly over time in both the deprived and non-deprived animals. VP content in the target brain region, the NTS, was significantly reduced in deprived animals following 23 h of fluid deprivation compared to non fluid deprived animals. The VP decrease in deprived animals reversed over time during which VP content in the NTS increased significantly at the Trial 1 and Ext Day behavioral time points. A similar trend of an increase in VP content in the NTS occurred in the non-deprived animals. These data from the BNST and NTS indicated that the fluid deprived animals were capable of increasing VP content and further indicated that failure to mount a rise in VP content in the PVN and AMe was not due to a deficit in VP synthesizing capability. Lastly, deprived and non-deprived animals exhibited similar VP content in two VP target sites in the LS and MS and in the control brain region, the IC.

Collectively, results of this investigation demonstrate that VP content changes in select brain regions during the maintenance phase of a CTA. Animals that maintained the CTA exhibited significantly higher VP content in the PVN and AMe

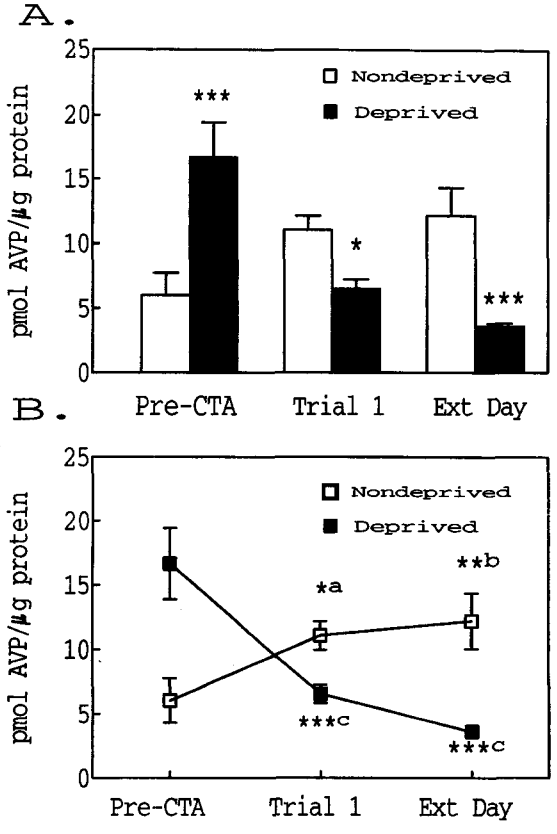

Fig. 4. Changes in VP content in the medial amygdala (AMe) between deprived and non-deprived animals (A) and within groups across behavioral time points (B). Data are presented as mean (±SEM) fmol VP/µg protein in the AMe of non-deprived and fluid deprived Fischer 344 male rats after a 23 h period before any conditioned taste aversion tests (Pre-CTA), after the first extinction trial (Trial 1), and at the day of extinction for deprived animals (Ext DAY) but when the non-deprived animals continued to maintain the CTA ($N = 68$). (A) A significant difference between deprived ($n = 11$) and non-deprived animals ($n = 11$) at the day of extinction (*$P < 0.05$). (B) A significant increase in VP content of the AMe occurred over time in the non-deprived group at the final day of extinction testing ([a]significantly different from non-deprived Trial 1, *$P < 0.04$). No change in VP content occurred over time in the fluid deprived animals.

whereas animals that did not maintain the CTA exhibited significantly lower VP content in the PVN and AMe. The lower VP content in the fluid-deprived, fast extinguishing animals was not due to a decrement in VP content relative to that observed at the Trial 1 time point, but was due to a failure to increase the VP content during the maintenance phase. Data from this study suggest that maintenance of a learned behavior is a neurochemically active process and is not the result of a passive storage system but instead requires a dynamic response that is, in this case, exhibited by a significant increase in VP levels in distinct regions of the rat brain indicating that maintenance of a learned association is an active process that requires changes in neurochemical systems involved in learning and memory.

Summary and future directions

From the outset we have sought to understand the mechanisms by which VP can enhance memory function and in the process determine whether VP fulfills the requirements for neurotransmitter status. The latter goal of proving the neurotransmitter status of VP has largely been achieved. With respect to the first goal, results of our work have shown that VP and its receptors are present in brain regions known to be involved in memory function, that release of VP is inhibited by a factor that inhibits memory function, that VP can significantly enhance the morphological complexity and outgrowth of neurons involved in memory function, that second messenger systems held to be involved in learning and memory, cyclic AMP and calcium signaling pathways, are potentiated and activated by VP, that electrophysiological models of memory function are induced by VP, and that when animals remember a learned association VP content in brain increases over time during the active phase of remembering. Collectively, these studies have taught us a great deal about the sites and mechanisms of VP action and have led us to pursue avenues of investigation that we would not have imagined 15 years ago when we began this work. We stand on the threshold of a new era in our research as we begin our studies of the role VP receptors play in the cerebral cortex. Thus far, results of these studies are quite exciting and promise to yield fascinating insights into the complexities of VP action in the most highly developed region of the mammalian brain, the cerebral cortex, the site of abstract

reasoning, judgment, complex analysis and the repository of those memories that last a life-time.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 3.1.4

Release of vasopressin within the brain contributes to neuroendocrine and behavioral regulation

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In addition to its peripheral secretion from the neurohypophysis, the neuropeptide vasopressin (VP) is released within the mammalian brain from probably all parts of the neuronal membrane. In particular the development of brain microdialysis in vivo together with blood microdialysis or blood sampling provides the advantage of being able to reliably compare the dynamic release patterns into different compartments of the organism. The central VP release within hypothalamic (e.g., supraoptic, paraventricular and suprachiasmatic nuclei) and limbic (e.g., septum, amygdala) rat brain areas is stimulated by a variety of substances and stressors, including interleukin-1β, social defeat and forced swimming. Furthermore, it is characterized by positive and negative feedback mechanisms and the capacity of the VP system for co-ordinated or independent release, the latter being observed, for example, during social defeat. This emotional stressor, in contrast to exposure to a novel cage, increased VP release within the supraoptic nucleus, but not into plasma. This failure to release VP peripherally could be observed also during forced swimming, despite a dramatic rise in plasma osmolality and a markedly stimulated central release. In another series of experiments we studied the effects of centrally-released VP on cognitive and emotional aspects of behavior using reverse microdialysis for antagonist administration during the behavioral tests and antisense targeting to downregulate either VP or its local V_1 receptor subtype. In this way, centrally (in particular septally) released VP could be shown to be causally involved in short-term memory and anxiety-related behavior. Furthermore, VP release within the hypothalamic paraventricular nucleus is likely to provide a negative tonus on the activity of the hypothalamic-pituitary-adrenocortical axis. This neuroendocrine effect together with cognitive, emotional and immunological effects of centrally released VP is thought to be essential to ensure adequate behavior of the animal during challenging situations and to contribute to the development of efficient coping strategies.

Introduction

Since the late 1960s, studies of central nervous system activities of pituitary peptides, among them vasopressin (VP), have been inspired by the pioneering work of David De Wied. Of all these peptides, VP has been one of the most widely studied, and major breakthroughs in neurobiological research covering areas from genes to behavior have been achieved by using VP as a 'model' neuropeptide. Although often controversial, ambiguous and difficult to interpret in a physiological context, previous more pharmacologically-oriented studies of central nervous system effects have not only helped to establish the neuropeptide concept (De Wied, 1983; De Wied et al., 1988) but have also resulted in a broad understanding of the complex involvement of endogenous neuropeptides in neuroendocrine, autonomic, immunological and behavioral mechanisms including their interactions. To pursue and extend this line of research, therefore, not only provides novel and fascinating insights into the regulatory phenomena of the mammalian brain, but also a means of appreciating David De Wied's original work.

VP fulfils all the requirements of a biologically potent neuropeptide (Landgraf, 1995). Predomi-

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nantly synthesized in magno- and parvocellular neurons of the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei, it is transported axonally to both the posterior pituitary and the external zone of the median eminence. The peripheral release into blood finally results in a broad spectrum of peripheral effects including antidiuresis and regulation of ACTH secretion from the adenohypophysis. Intracerebrally released into the extracellular fluid from probably all parts of the neuronal membrane, VP as a neuromodulator/neurotransmitter triggers a variety of central effects on neuroendocrine, autonomic, immunological, emotional and cognitive functions. The multiplicity, diversity and physiological impact of these effects are hard to understand without more information about the anatomical and functional characteristics of the central vasopressinergic system. Points that remain unclarified include: (1) the coordination of VP release from dendrites, perikarya, axons en passant and axon terminals into different compartments; (2) the differential contribution of magno- versus parvocellular neurons and of the SON versus the PVN to certain neuropeptide effects; and (3) the synergistic or even opposing effects of VP released within distinct brain areas or/and into the systemic circulation. Using microdialysis, antisense targeting and a variety of other molecular and cellular approaches in conjunction with ethologically relevant behavioral tests, our laboratory is trying to help answer these questions by focusing on potential stimuli, mechanisms and physiological consequences of central VP release.

Regulation of VP release within the brain and into the blood

It is important to realize that neuropeptides such as VP only become biologically active after their release into the extracellular space. To measure this release, the majority of previous studies have relied on post-mortem methods or single collections, which only offer limited snapshots at selected time frames in a dynamic process. Thus, although measurements of VP content in brain tissue, of VP concentrations in the cerebrospinal fluid and attempts to reflect central fluctuations in VP just by determining plasma VP have all substantially contributed to our present knowledge, they have to be interpreted with extreme caution.

Microdialysis has rapidly become popular in recent years as an in vivo approach to monitor changes in neuropeptide concentrations in the brain's extracellular fluid. The development of simultaneous brain and blood microdialysis for VP and oxytocin (OT) (Neumann et al., 1993a; Ludwig et al., 1994a) provides the advantage of being able to reliably compare the dynamic release patterns into the different compartments and thus to elucidate the capacity of the vasopressinergic system for independent or coordinated release. Furthermore, microdialysis in freely moving animals provides the opportunity to correlate neuropeptide release with ongoing behavior, one of the major goals in neuroendocrine research. It also allows the use of inverse microdialysis (retrodialysis) approaches to simultaneously administer exogenous substances, including antagonists, without acutely disturbing the animal's behavior. Technical advantages include the exclusion of large molecules (e.g., enzymes or peptides that could interfere with the radioimmunoassay) and the minimal damage to the tissue, including the blood–brain barrier, compared to push-pull perfusion. Nevertheless, in view of the dimensions of the probe and the rat brain, the extent of damage should not be ignored and experiments have to be carefully designed to show up neuroendocrine consequences and behavioral deficits, which could be attributed to the implantation per se. Major disadvantages of microdialysis include the low recovery and, due to the consequent duration of collection intervals, a relatively poor time resolution. Nevertheless, in terms of temporal and spatial characteristics of release, distribution, enzymatic degradation and re-uptake processes, neuropeptide microdialysis data should be more representative and reliable than those of 'classical' transmitters. By dialyzing a relatively large area (approx. 1 mm^3), microdialysis necessarily 'integrates' temporal as well as spatial fluctuations and gradients, but this certainly creates fewer problems for the analysis of volume than of wired transmission (see Fuxe and Agnati, 1991).

Probably the simplest approach to study central release patterns within distinct brain areas with

minimal external input is to change the composition of the microdialysis fluid. The direct effect on VP neurons of altered osmolality, addition of depolarizing or release-inhibiting agents and omission of Ca^{2+} has been widely used to monitor subsequent effects on local and peripheral release patterns. The release of VP within the hypothalamic nuclei occurs predominantly from dendrites and perikarya (Pow and Morris, 1989; Morris and Pow, 1993). As shown by di Scala-Guenot et al. (1987) in vitro and Ludwig et al. (1995) in vivo, it is not prevented by tetrodotoxin, which selectively blocks the sodium-dependent generation and propagation of action potentials. Hence, release from axon collaterals or terminals shown to be present in the hypothalamic nuclei (van den Pol, 1982; Mason et al., 1986; Ray and Choudhury, 1990) either does not play a major role or, although relaying significant information, is simply not detectable by microdialysis. Typical changes in release during and after addition of depolarizing agents or after omission of Ca^{2+} suggest active exocytosis from intact neuronal structures rather than 'outflow' from damaged neurons. This is further confirmed by a series of experiments all indicating that VP release may be modulated over a wide range. Among others, interleukin-1β, angiotensin, glutamate and the $GABA_A$ receptor antagonist bicuculline were shown to facilitate VP release within the PVN and SON. In contrast, inhibitory effects were exerted by the glutamate antagonist kynurenic acid and the V_1/V_2 receptor antagonist $d(CH_2)_5Tyr(Et)VP$ (Table 1). The latter finding is particularly interesting as it indicates the potential of locally-released VP to feed back on the neurons of its origin in a positive manner. This action is reminiscent of similar effects of endogenous VP observed in the septum in vitro (Landgraf et al., 1991a). Compared to OT, where the intra-SON feedback action is likely to be involved in reproduction-related processes (Neumann et al., 1996) and morphine withdrawal excitation of OT neurons (Brown et al., 1997), the physiological significance of the positive VP feedback action is largely unknown. In concert with other mediators, it probably ensures a local amplification cascade, which underlies the adequate intranuclear response to a stressful challenge and the involvement of intranuclear VP in cognitive processes and neuroendocrine regulation. Once the local VP concentration reaches a threshold sufficient to increase intracellular Ca^{2+} and thus to hyperpolarize the neurons (Ludwig and Leng, 1997), their phasic firing and, consequently, the peripheral release of VP into the circulation would be inhibited. So far, however, we did not succeed in stimulating peripheral VP release by intracerebroventricular or intra-PVN administration of a V_1 receptor antagonist. Future studies will have to focus on the contribution of both positive and negative feedback mechanisms to the regulation of central and peripheral release patterns.

As shown by cross-talk studies in female rats, the vasopressinergic system, compared to the oxytocinergic one, is not similarly subject to lactation-related plasticity (Neumann et al., 1995). An involvement of multisynaptic pathways in regulating intrahypothalamic VP release is suggested by tetrodotoxin treatment and by lesion of the AV3V region both of which resulted in an abolition of the central neuropeptide response to systemic osmotic stimulation (Ludwig et al., 1995, 1996a).

It is worth mentioning that the concentration of VP in the hypothalamic extracellular fluid may be orders of magnitude higher than that in plasma (Landgraf, 1992). In the light of this concentration gradient, the often discussed question of whether plasma VP is likely to penetrate the blood–brain barrier is therefore unsubstantiated. Even small amounts of plasma VP reaching the brain would probably be without any physiological significance as it is rather the local release pattern and local fluctuations in VP concentrations that trigger physiological effects in the brain. Also a slight 'leakage' of central VP to plasma (Banks et al., 1987) is unlikely to be physiologically relevant. Hence, as far as the hypothalamic-neurohypophysial system and endogenous VP specifically is concerned, the blood–brain barrier separates the compartments in a relatively strict manner. Its development, however, makes sense in evolutionary terms only if the VP neurons took advantage of this compartmentalization by developing the capability of releasing VP from different parts of their plasmalemma in an increasingly differentiated manner. The capability to independently release VP within the brain and into the systemic circulation

Table 1
Examples of intracerebral release of VP in the rat in vivo in response to various stimuli[a]

Stimulus	Administration	Area	VP release	References
1. Osmolality				
Hypertonic NaCl	Via MD	SON, PVN	Increase	Landgraf and Ludwig, 1991; Ludwig and Landgraf, 1992; Neumann et al., 1993a; Ludwig et al., 1994a
Hypertonic mannitol, urea	Via MD	SON	Increase	Ludwig et al., 1994b
Hypotonic NaCl	Via MD	SON	Increase	Ludwig and Landgraf, 1992
Hypertonic NaCl	i.v., i.p.	SON	Increase (delayed)	Ludwig et al., 1994a Neumann et al., 1993a, 1995
	i.v.	PVN	Increase	Ota et al., 1994a
	i.p.	Septum, hippocampus	Increase	Demotes-Mainard et al., 1986; Landgraf et al., 1988
2. Peptides/antagonists				
Interleukin-1β	Intranuclear	SON	Increase	Landgraf et al., 1995c
		PVN	Unchanged	
	Via push-pull Perfusion	PVN	Increase	Watanobe and Takebe, 1993
Cholecystokinine-8	Intranuclear, iv	SON	Increase	Neumann et al., 1994a
Angiotensin	i.c.v.	SON, PVN	Increase	Moriguchi et al., 1994
V1/V2 antagonist	Intranuclear	SON	Decrease	Wotjak et al., 1994
		SCN	Unchanged	Kubota et al., 1996
Naloxone	i.v.	SON, NTS	Unchanged	Russell et al., 1992
		Septum, hippocampus	Unchanged	Neumann et al., 1991
Bicuculline	Via MD	SON	Increase	Landgraf et al., 1995b
Kynurenic acid	Via MD	SON	Decrease	Landgraf et al., 1995b
3. Other agents				
K^+	Via MD	SON, PVN	Increase	Ludwig and Landgraf, 1992; Landgraf et al., 1992; Neumann et al., 1993b
		Medial amygdala	Increase	Landgraf et al., 1995b
		BNST	Increase	Landgraf et al., 1995b
		SCN	Increase	Kubota et al., 1996
Ca^{2+}-free + EGTA	Via MD	SON, PVN	Decrease	Ludwig and Landgraf, 1992; Landgraf et al., 1992; Neumann et al., 1993b
Tetrodotoxin	Via MD	SON	Unchanged	Ludwig et al., 1995
Carbachol	Via MD	PVN	Increase	Ota et al., 1992
4. Reproduction				
Pregnancy		Septum	Increase	Landgraf et al., 1992
		Hippocampus	Unchanged	Landgraf et al., 1992

Table 1 (*continued*)

Stimulus	Administration	Area	VP release	References
Parturition		SON, PVN	Unchanged	Landgraf et al., 1992; Neumann et al., 1993b
		Septum, hippocampus	Increase	Landgraf et al., 1992
Suckling		SON, PVN	Unchanged	Neumann et al., 1993b, 1995
		Septum, hippocampus	Unchanged	Neumann and Landgraf, 1989; Landgraf et al., 1992
5. Other manipulations				
Hemorrhage		PVN	Increase	Ota et al., 1994b
		Septum	Increase	Demotes-Mainard et al., 1986
SON stimulation	Via MD	Median eminence	Increase	Wotjak et al., 1996a
	Via MD	Septum	Increase	Engelmann et al., 1994
	Electrical	Septum	Increase	Demotes-Mainard et al., 1986
PVN stimulation	Via MD	Median eminence	Unchanged	Wotjak et al., 1996a
	Electrical	Septum	Increase	Neumann et al., 1988
	Electrical	NTS	Increase	Landgraf et al., 1990a
Emotional stress		PVN	Increase	Wotjak et al., 1996b
		SON	Unchanged	Wotjak et al., 1996b
Physical stress		PVN, SON	Increase	Wotjak et al., 1998
Fever		Ventral septum	Increase	Landgraf et al., 1990b; Chen et al., 1997
		Hippocampus	Unchanged	Landgraf et al., 1990b
AV3V lesion		SON	Decrease	Ludwig et al., 1996a

[a] Intranuclear administration means that the respective peptide was given via a fused silica capillary directly into the dialysed nucleus. Abbreviations used: BNST bed nucleus of the stria terminalis; i.p., intraperitoneal; i.v., intravenous; MD, microdialysis; NTS, nucleus tractus solitarii; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus.

could, in turn, only result in differentiated and multiple effects if the compartments became separated by a barrier. Therefore, probably in a co-evolved manner, both the blood–brain barrier and the capacity of neuropeptidergic neurons developed to increasingly involve neuropeptides in central and peripheral functions. Only then could a broad spectrum of central and peripheral effects be established, which are either not necessarily interrelated or are closely interrelated in a synergistic manner. In order to test this hypothesis, we were looking for stimuli useful in studying the capacity of neurons in the SON and PVN to independently or simultaneously release VP within the brain or/and into the systemic circulation.

Our first approach was to simply increase or decrease the osmolality of the dialysis medium. In contrast to K^+, which mediates its effects directly by passing through selective ion channels (Ludvig et al., 1994), changes in extracellular fluid osmolality caused by dialysis with hypertonic sodium might induce neuronal depolarization indirectly, for instance via mechanosensitive stretch-inactivated channels (Oliet and Bourque, 1993). The latter have only been observed in magnocellular neurons of the SON so far. Whether these channels also exist in parvocellular VP neurons remains to be determined. The typical pattern of intra-SON release of VP includes a very robust rebound phenomenon after osmotic stimulation with sodium

and a strong response to a decrease in osmolality (Ludwig and Landgraf, 1992). Under these conditions, central release is not always paralleled by peripheral release. This finding was confirmed later after intraperitoneal or intravenous administration of hypertonic sodium (Neumann et al., 1993a; Ludwig et al., 1994a). In contrast to direct osmotic stimulation of the SON via microdialysis, intra-SON release in response to systemic osmotic stimulation is delayed and long-lasting, generally occurring at a time when plasma VP has returned to baseline levels (Fig. 1). In this context it is worth mentioning that an acute systemic osmotic challenge also produced an instant increase in VP release in the septum and hippocampus (Landgraf et al., 1988), suggesting that stimulatory osmotic mechanisms triggering release from axon terminals located in the posterior pituitary or limbic brain areas may differ from those inducing intrahypothalamic release. Indeed, in contrast to the sodium-dependent peripheral release into the systemic circulation after direct stimulation of the SON, intra-SON release of VP seems to depend primarily on local changes in osmolality rather than in sodium concentrations (Ludwig et al., 1994b). Differences in mechanisms underlying central and peripheral VP release are further substantiated by the finding that salt loading (2% sodium for 2 days) abolished the intra-SON response of VP to osmotic stimulation, whereas VP release into the systemic circulation was markedly elevated (Ludwig et al., 1996b). Another example of independent central and peripheral release patterns is the administration of interleukin-1β or emotional stress (see below). The regulatory capacity of differentiated release would enable VP to elicit behavioral effects, for example, without necessarily triggering antidiuresis, thus facilitating the involvement of an endogenous neuropeptide by a priori quite different mechanisms. Another interesting aspect is the possibility that this regulatory potential also applies to a single neuron. Thus, SON neurons are apparently capable – dependent on the quality and intensity of a given stimulus – of regulating their dendritic and perikaryal (i.e., intranuclear) and axonal (i.e., peripheral) release of VP in either an independent or coordinated manner. Future studies into this remarkable ability are needed.

To elucidate the regulation of release into different and separate compartments, the release dynamics under basal and stimulated conditions have to be monitored over relatively long periods of time (see Fig. 1). Only then, possibly during the same response pattern, intervals of independent as well as simultaneous release can be observed. Although more intensely studied in the oxytocinergic system, VP may be simultaneously released within the brain and into the systemic circulation, also during certain time intervals following osmotic stimulation. This coordinated release is probably the prerequisite for integrative, synergistic central and peripheral effects, whereas the independently regulated release may serve rather selective and specific actions in different compartments of the organism. Under physiological conditions, probably only the interplay between both modes of release enables the organism to adequately cope with internal and external challenges.

After having established simultaneous microdialysis in the SON/PVN and in the blood, we tried to monitor VP release in other hypothalamic and extrahypothalamic areas as well. Because of the minute amounts released in the latter, this attempt turned out to be a methodological challenge. Its necessity results from the fact that the push-pull perfusion procedure used previously sometimes shows problems in balancing inflow and outflow of the perfusate and, therefore, should not be performed in conscious, freely behaving animals.

In the suprachiasmatic nucleus, the primary pacemaker for generation and entrainment of circadian rhythms located in the anterior hypothalamus, a daily variation in VP release with a pronounced peak during subjective daytime and a trough around midnight could be shown (Kalsbeek et al., 1995). Although only about one third of the amount of VP usually recovered in the SON and PVN was found in this nucleus, there is convincing evidence of an intranuclear source of VP. Firstly, the amount of VP detected in microdialysates was clearly correlated with the distance of the probe to the suprachiasmatic nucleus (Kubota et al., 1996). If the dialysis membrane was placed more than approx. 200 μm caudal or lateral to the nucleus, the recovery was drastically reduced. This finding, in the meantime hundredfold confirmed in our laboratory also in the

SON and PVN, is an important argument for an intranuclear origin of centrally-released VP. Neuropeptide diffusion over long distances is further excluded by the fact that OT was detectable only in dialysates collected from both the SON and PVN, but not the suprachiasmatic nucleus. Secondly, in contrast to the suprachiasmatic nucleus, neither the SON nor the PVN showed any signs of daily variations in VP release (Kalsbeek et al., 1995). In a follow-up study, VP release within the suprachiasmatic nucleus could be shown to be responsive to high K^+solution or direct osmotic stimulation, indicating release from intact neuronal structures (Kubota et al., 1996). The latter may be axon terminals, dendrites and somata, as recently demonstrated by Castel et al. (1996). To determine the precise mechanism of VP release within the suprachiasmatic nucleus, further studies are required, including retrodialysis with tetrodotoxin during concomitant pharmacological stimulation (Ludwig et al., 1995), thus blocking the propagation of action potentials and, consequently, of axonal release. Interestingly, in contrast to the SON and septum, VP released within the suprachiasmatic nucleus fails to influence its own release (Kubota et al., 1996). Administration of a combined V_1/V_2 receptor antagonist directly into the dialyzed area had no effect on the intranuclear release of VP during basal and stimulated conditions. Thus, autoregulatory mechanisms of VP release probably do not contribute to the intrinsic capability of the suprachiasmatic nucleus to generate a circadian rhythm in VP release.

Although changes in VP release are more difficult to determine in extra-hypothalamic regions than in hypothalamic production areas, this kind of information is important to obtain, particularly in the context of neuropeptide release and behavioral performance. Despite the radioimmunoassay used in conjunction with microdialysis being extremely sensitive (<0.1 pg/dialysate), basal VP release in the medial amygdala and the bed nucleus of the stria terminalis of sham-operated male, castrated male, and female rats was hardly detectable, even if 60-min instead of 30-min dialysates were collected. Addition of a depolarizing agent (56 mM K^+) to the dialysis medium, however, resulted in a rise in VP dialysate concentrations suggesting that a releasable pool in the amygdala may respond to appropriate stimulation. The corresponding increase in VP release was higher in sham-operated males than in female or castrated male animals (Landgraf et al., 1995b). In the bed nucleus of the stria terminalis, basal release of VP was also found to be increased during K^+ stimulation, further confirming microdialysis as a tool to monitor neuropeptide release even in limbic brain areas, at least under stimulated conditions. Finally, we tried to push-pull perfuse the area containing the median eminence using an angled approach to avoid damage to the sagittal sinus by the implanted probe (Wotjak et al., 1996a). Although there was considerable damage to the median eminence, this method in conjunction with microdialysis of the SON and PVN proved to be effective in revealing interactions between the hypothalamic-neurohypophysial system and the hypothalamic-pituitary-adrenal (HPA) axis which might have an impact on the response to selected stressful stimuli (see below).

Emotional and physical stressors trigger central VP release: VP like OT is a stress neuropeptide in the rat

If endogenous VP really is involved in stress-coping strategies, it should be released centrally in response to emotional or physical stressors. So far, both VP and OT have almost exclusively been measured in plasma and cerebrospinal fluid. The hypothesis drawn from these studies that 'oxytocin unlike vasopressin is a stress hormone in the rat' (Lang et al., 1983) needs to be extended and updated in the light of recent findings.

One approach we used monitored VP release within the PVN as this nucleus orchestrates the HPA axis, integrating endocrine, autonomic, immunological and behavioral responses to stress. We focused strictly on ethologically relevant and naturally occurring stressors, as only these can reveal the physiological role VP plays in challenging situations. The social defeat paradigm we used consisted of placing the dialyzed animal in the home cage of a dominant male resident that had been trained to be aggressive towards intruders. Immediately after the first attack, the intruder was

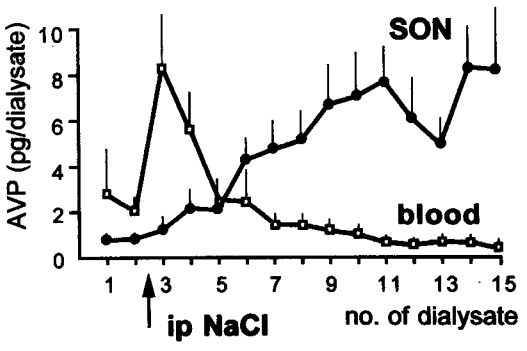

Fig. 1. VP content in 30-min dialysates sampled simultaneously from the SON and blood of female urethane-anesthetized rats. Before starting the third dialysis interval, hypertonic saline was administered intraperitoneally. Significantly different from pre-stimulation: dialysate 3 (blood), from dialysate 4 (SON). Modified from Neumann et al. (1995).

physically separated from the resident by a wire mesh, still allowing olfactory and visual contact. Social defeat was considered successful if the intruder showed submissive body postures (Koolhaas et al., 1980) or freezing behavior for at least 15 min during the 30-min stress exposure. As control, dialysates were collected from rats placed in the same cages with the inserted wire mesh, but without the resident. While VP release under basal conditions was comparable in the two groups, social defeat caused a significant rise during and after emotional stress (Fig. 2) (Wotjak et al., 1996b). This response is remarkably specific, as VP release within the SON and that of OT within the PVN were not changed under identical experimental conditions. Exposure to the novel cage, in contrast, failed to alter VP release within the PVN. Both social defeat and novelty resulted in an increase in plasma ACTH and corticosterone, the former stressor tending to trigger higher concentrations. Unchanged plasma lactate levels before and during social defeat indicated the dominance of emotional components. Interestingly, neither plasma VP nor OT responded to the stressor (Wotjak et al., 1996b), further confirming the regulatory capacity of the system to differentially release VP into different compartments. This paradigm appears to reflect the evolutionary advantage mentioned before: VP released centrally is now available to ensure an adequate behavioral performance and to contribute to coping strategies; the homeostasis, particularly the water metabolism, is apparently not acutely threatened by the stressor used.

As demonstrated in another approach, forced swimming over a 10-min period, a combined physical and emotional stressor has more "global" effects since it markedly increased both plasma lactate and osmolality. Under these experimental conditions, VP release in both the PVN and SON was increased significantly (Wotjak et al., 1998). Again, plasma VP remained virtually unchanged. With regard to the close relationship between plasma osmolality and plasma VP concentration (Dunn et al., 1973; Windle et al., 1996) the failure to release VP into systemic circulation despite the marked rise in plasma osmolality (by 16 mOsm/kg H_2O) is remarkable and remains to be elucidated. Considering the enormous amount of VP stored in the posterior pituitary it is unlikely that it reflects the priority of central release. In contrast to VP, plasma OT was significantly elevated during forced

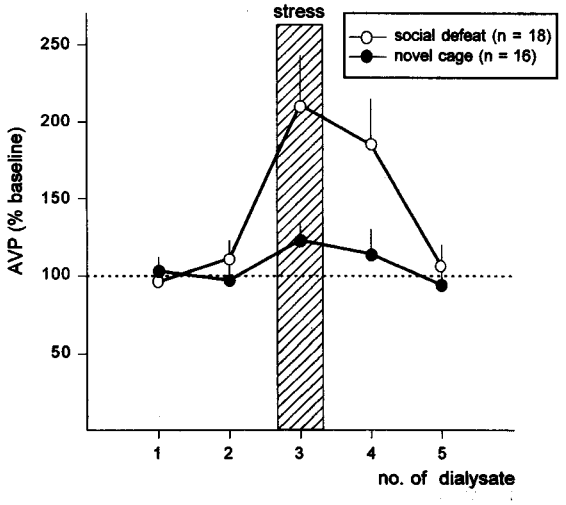

Fig. 2. Effect of emotional stress on VP release within the PVN of freely moving male rats. Data are expressed as percentage of baseline (100%, dotted line). Animals were exposed to a dominant resident (social defeat) or to a novel cage during the third dialysis interval. Significantly different from pre-stimulation: dialysate 3 and 4 (social defeat). Modified from Wotjak et al. (1996b).

swimming, further confirming that the capability for independent central and peripheral release applies more to VP than to OT. Neither the underlying mechanisms nor the functional implications of this striking difference between VP and OT are well understood. In this context, two preliminary observations are worth to be mentioned: Firstly, simulation of peripheral secretion from the posterior pituitary by intravenous infusion of synthetic AVP failed to influence the coping strategy of the animals during forced swimming (e.g., struggling versus floating). Secondly, even retrodialysis administration of bicuculline into the PVN during swim stress did not result in a rise of plasma AVP, making a contribution of GABA to the blockade of AVP secretion unlikely (Engelmann, unpublished observations).

It was probably the differential neuropeptide response to stimulation that led Lang et al. (1983) to suggest that OT, unlike VP, is a stress hormone in the rat. Although this may be true for plasma (see also Samson and Mogg, 1990), our data suggest the opposite, namely, that in the brain, VP rather than OT is a stress neuropeptide. Without any doubt, more than just plasma has to be measured in order to draw any conclusion about stress-related alterations in neuropeptide release. As soon as spatially and temporally differentiated release pattern of a given neuropeptide, its stimulus-dependency, receptor characteristics etc. are demonstrated, such generalizations are certainly of limited value and are sometimes a temptation to interpret neuroendocrine processes in a simpler way than is necessary.

Effects of centrally-released VP on cognitive and emotional aspects of behavior

As behavioral responses to stress are extremely complex, we chose relatively simple and naturally-occurring tests to uncover the impact of centrally-released VP on cognitive and emotional parameters. These effects are mediated by VP receptors present inside and outside the hypothalamic nuclei (Ostrowski et al., 1994). Ideally, physiological and behavioral consequences should be studied in the same test situation – one in which central release of VP has been demonstrated.

Social recognition/discrimination for investigating short-term memory processes is based on the rat's olfactory discriminative capacities and has the advantage that, due to the animal's innate desire to communicate with conspecifics, no artificial stimulus has to be used. Social recognition is usually measured by the time adult animals spend investigating juvenile ones. When the same juvenile is reexposed to the adult 30 min after its initial exposure, the adult rat displays recognition of this juvenile as indicated by a significantly reduced investigation duration during the second exposure. Engelmann et al. (1995) refined this paradigm by simultaneously presenting a novel juvenile together with the previously encountered one during the second exposure. This simultaneous presentation has several advantages as it provides an internal control under identical experimental conditions and the opportunity to clearly distinguish between specific and unspecific effects. Furthermore, in general it copes better with the remarkable cognitive performance of rats.

To stress rats emotionally, social defeat in conjunction with elevated plus-maze exposure is used in our laboratory. As already mentioned, the procedure is to place a rat in the home cage of a dominant resident that has been trained to be aggressive towards intruders. Immediately after defeat, rats are usually tested in the elevated plus-maze (Liebsch et al., 1995). This test is based on the creation of a conflict between the exploratory drive of the rat and its innate fear of open, exposed and elevated areas. Reduced open-arm exploration serves as an index of increased anxiety-related behavior. In the rat, anxiety is the best understood emotion and has been shown to be closely linked to learning and memory processes (LeDoux, 1995).

We used social recognition/discrimination and made sure that this paradigm critically involves VP. It indeed does, since the juvenile-specific olfactory information is stored for at least 2 h, if 1 ng VP is given intracerebroventricularly; in turn, if the V_1 receptor antagonist $d(CH_2)_5Tyr(Me)VP$ is administered, the rat remembers the conspecific juvenile for less than 30 min (Landgraf et al., 1995a). Similarly, administration of the V_1 antagonist into the septum of Long Evans rats by retrodialysis impaired the social recognition of these animals (Engelmann

and Landgraf, 1994). After this kind of treatment, their behavioral performance was comparable to that of homozygous Brattleboro rats that are not able to synthesize biologically active VP in their hypothalamic nuclei due to a frameshift mutation in the corresponding gene (Schmale and Richter, 1984). Retrodialysis administration of synthetic VP, on the other hand, improved social recognition abilities in both rat strains. The next experimental step was to use direct osmotic stimulation of the SON by microdialysis to stimulate intranuclear VP release and to simultaneously measure both VP release and the social recognition ability of the dialysed rat. Again, the microdialysis procedure per se failed to disturb the behavioral performance as controls did not differ from untreated rats. The stimulated intra-SON release of VP was associated with a better performance in the social memory test (Engelmann et al., 1994). Addition of the V_1 antagonist to the dialysis medium abolished this effect indicating a critical involvement of endogenous VP. As shown in a follow-up study, the rise in intra-SON release was accompanied by an increased release of VP in the septum, suggesting the possibility of coordinated release patterns in hypothalamic and limbic brain areas even in the absence of direct fiber connections (Engelmann et al., 1994). The contribution of septal release of VP during SON stimulation to the improved short-term memory has to be shown. In any case, the SON is certainly more than just a nucleus projecting to the posterior pituitary and contributing to the peripheral release of VP.

Although the use of receptor antagonists is more physiological than, for instance, administration of the synthetic agonist, it bears certain risks that have to be acknowledged. Many neuropeptide receptor antagonists are known to cross-react with more or less closely related receptors, induce receptor hypersensitivity or have partial agonist-like activity. Therefore, the involvement of a given neuropeptide can generally be established only by combining different approaches. In this context, a more rational strategy would be to block the synthesis of either the endogenous ligand or its receptor protein rather than to inhibit the ligand-receptor interaction by antagonists. Antisense targeting fulfils this requirement. This promising but challenging tool (Landgraf, 1996; Landgraf et al., 1997) is based on antisense oligonucleotides thought to inhibit the synthesis of a given neuropeptide or receptor protein by downregulating the intracellular mechanisms involved in its production.

In a series of experiments we tried to reveal the physiological significance of centrally-released VP by inhibiting its local synthesis. A VP mRNA antisense oligonucleotide infused bilaterally into the SON of lactating rats failed to affect either OT release into blood or milk yield to the pups, both parameters being significantly reduced in animals treated with an OT antisense oligonucleotide (Neumann et al., 1994b). Whereas this finding could be expected from a functional point of view, failures under similar experimental conditions include the unchanged water consumption and urine osmolality, even though these effects had been observed in our laboratory after intracerebroventricular administration (Skutella et al., 1994). We do not know the reasons for these failures, but would like to issue a word of caution as regards the sole use of antisense oligonucleotides targeting neuropeptide mRNA and behavioral testing of animals treated this way.

An alternative antisense approach to study the physiological impact of centrally-released VP is to downregulate the receptor subtype protein instead of the neuropeptide. Although acute central actions mediated by the VP system are more likely to be due to changes in central VP release than to changes in receptor characteristics (Landgraf et al., 1991b), its comparatively slow turnover might make the VP receptor a promising target for antisense approaches. Accordingly, an antisense oligonucleotide directed towards the V_1 receptor subtype mRNA was infused into the septum via osmotic minipumps and a variety of molecular, cellular and behavioral parameters were determined (Landgraf et al., 1995a). Compared to vehicle and scrambled sequence controls, chronic antisense administration for up to 4 days not only significantly reduced septal V_1 receptor density, but also the capability of the rats to recognize previously encountered juveniles, confirming the behavioral impact of VP-receptor interactions in this limbic brain area. Furthermore, in contrast to control animals, antisense-treated rats failed to respond to

the memory-improving effect of 1 ng synthetic VP administered intracerebroventricularly. This relationship between ligand-receptor interaction and behavioral performance is confirmed by a positive correlation between social recognition abilities on the one hand and the amount of intra-SON-released VP and V_1 receptor density in the septum on the other (Fig. 3).

As cognitive and emotional components are closely interrelated, we were interested in gaining more insight into VP effects on anxiety-related behavior, all the more so, as we used fear-related stimuli to increase central VP release. V_1 receptor antisense-treated animals in the above-mentioned study clearly showed signs of reduced anxiety as they made more entries into and spent more time on the open arms of the elevated plus-maze (Landgraf et al., 1995a). This finding has recently been confirmed by administration of the V_1 receptor antagonist into the septum of rats (Liebsch et al., 1996). As mentioned already, both the antagonist approach and the antisense technology have their own limitations and pitfalls; hence 'key' results should be confirmed by the respective alternative.

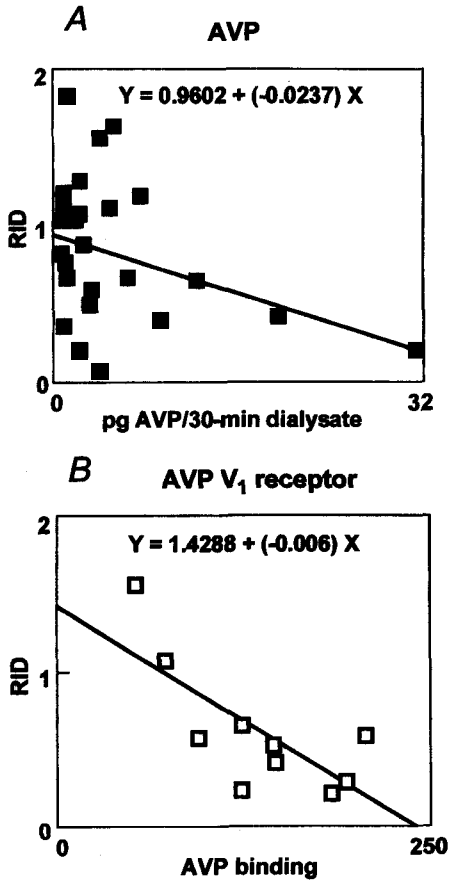

Fig. 3. Positive correlation between social recognition abilities in adult male rats (an improvement is indicated by a decreased ratio of investigation duration, RID) and (A) the amount of VP released intra-SON and (B) the number of VP V_1 binding sites within the lateral septum. Modified from Engelmann et al. (1996).

The described relationship between cognitive deficit on the one hand and reduced anxiety-related behavior on the other deserves attention. No less interesting is the issue of whether intra-PVN-released VP in response to emotional stress is critically involved in coping strategies during the stressful exposure. There are reasons to believe that VP, released during social defeat and acting as neuromodulator, is relevant for stressor-related emotional memory rather than for current coping strategies. Accordingly, preliminary experiments in our laboratory using intra-PVN administration of the V_1 receptor antagonist suggest altered behaviors not during the first stress exposure (i.e., during antagonist administration), but during those on the following day, when the animals were again socially defeated, but did not receive the antagonist. This finding suggests that VP released intra-PVN in response to an emotional stressor is likely to influence the storage/retrieval of stress-related information so that the animal can respond more adequately if it is exposed once again to the same or a similar stressor (Wotjak et al., 1996a; Ebner and Engelmann, unpublished observations). Thus, the fine-tuned central release pattern of VP in response to emotional and physical stressors apparently corresponds with the central effects of the neuropeptide to ensure adequate behavior of the animal during challenging situations. According to our experience, VP contributes substantially in retaining a 'normal' behavioral performance. This, however, does not necessarily mean that administration of synthetic VP results in an improvement of behavioral parameters (see Engelmann et al., 1992). There is no doubt that more has to be learned

about the interactions between emotionality and cognition. What is referred to as emotional memory is required for the acquisition and storage of information about the emotional significance of experiences. It is essential for learning about new dangers quickly, even, if possible, from a single experience, to enhance survival in future encounters with the same threat or others like it (Rogan and LeDoux, 1996). An endogenous neuropeptide such as VP that, as demonstrated, has the potential to increase anxiety-like behavior and to improve cognitive parameters, is likely to be a good candidate for involvement in emotional memory (for review see Engelmann et al., 1996). This hypothesis is further substantiated by the finding that Roman Low Avoidance rats had higher VP mRNA levels than Roman High Avoidance rats in the PVN under basal conditions, the former being more emotionally reactive. This increased VP expression may not only contribute to the regulation of anxiety-related behavior, but also participate in the mechanisms underlying the hyperactivity of the HPA axis characterizing the Low Avoidance line (Aubry et al., 1995).

Similarly, we recently succeeded in selectively breeding rats for anxiety (Liebsch et al., 1998). Animals with an inborn high anxiety-related behavior tended to express more VP in parvocellular neurons of the PVN than rats with a low anxiety-related behavior and to show signs of an activated HPA axis (Liebsch and Landgraf, unpublished observations).

Contribution of centrally-released VP to the regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis

The stress-induced release of VP within distinct brain areas raises the question of interactions with the HPA axis, particularly at the level of the PVN. This axis is of critical importance for homeostasis, as it minimizes deviations from the homeostatic state and helps to return to equilibrium after stress-induced disturbances (Munck et al., 1984). Although the volume transmission concept (Fuxe and Agnati, 1991) suggests diffusion of neuropeptide modulators to distant receptors, local autocrine/paracrine VP actions via receptors within the PVN or its perinuclear zone have to be taken into consideration. As the PVN is one of the most complex and heterogeneous nuclei in the mammalian brain, only rough attempts can be made to elucidate the physiological impact of a single neuropeptide being released intranuclearly. An additional complication is due to the fact that CRH is co-localized with VP in parvocellular neurons of the PVN and may be released intrahypothalamically in response to selected stimuli (Merlo Pich et al., 1993a). Furthermore, CRH and VP seem to interfere mutually with their release patterns (Bernardini et al., 1994) exerting, for example, synergistic effects on the investigative behavior of rats after intracerebroventricular infusion (Elkabir et al., 1990). Hence, we administered a cocktail containing both the V_1 and the α-helical CRH receptor antagonists by retrodialysis into the PVN and measured the effects on plasma ACTH (Wotjak et al., 1996b). Although this combined administration excludes a priori an unequivocal interpretation of the data, it prevents possible interference from, or even counter-regulation effects of, CRH. This kind of treatment resulted in a rise in ACTH secretion into the blood of resting animals and also during emotional stress, indicating an inhibitory effect of intra-PVN-released VP and/or CRH. There are reasons to suggest that this negative tonus is most likely attributable to the action of VP. Firstly, central CRH has a rather stimulatory effect on HPA axis activity (Ono et al., 1985; Antoni, 1993). Secondly, intracerebroventricular infusion of a VP, but not of a CRH, antagonist stimulates the release of CRH into portal blood, i.e., of the predominant secretagogue of ACTH under basal (Whitnall, 1993) and stimulated (Merlo Pich et al., 1993b) conditions. The finding that intra-PVN release of VP is likely to inhibit the release of ACTH secretagogues from the median eminence into portal blood and hence of ACTH from the adenohypophysis, adds to a picture of HPA axis regulation which is progressively becoming more complex. It is also becoming ever more clear that the role VP plays in this context depends on the site of release (Fig. 4): (1) Released from axon terminals of parvocellular neurons of the PVN, VP diffuses into portal blood at the level of the zona externa of the median eminence to stimulate ACTH secretion (Antoni, 1993). (2) As shown

in a recent combined microdialysis/push-pull perfusion study in the rat, VP is released en passant from axons of magnocellular neurons in the zona interna of the median eminence projecting to the posterior pituitary (Wotjak et al., 1996a) indicating that VP released in this way might mediate a communication between the hypothalamic-neurohypophysial and the HPA systems in response to selected stimuli. This finding confirms previous results (Buma and Nieuwenhuys, 1987; Nordmann and Dayanithi, 1988). (3) VP could reach the adenohypophysis via short portal vessels after its release from nerve terminals in the posterior pituitary (Bergland and Page, 1978). Furthermore, as shown in our own study (Wotjak et al., 1996b), VP originated and released within the PVN inhibits ACTH release under both basal and stressful conditions by as yet unknown mechanisms (Fig. 4, point 5). Additionally, vasopressinergic neurons of the suprachiasmatic nucleus project to the dorsomedial hypothalamus/PVN area where release of VP inhibits HPA axis activity, probably via a neural input to the adrenal gland (Kalsbeek et al., 1996) (Fig. 4, points 6 and 7).

This extension of the concept of HPA axis regulation including the involvement of the hypothalamic-neurohypophysial system and multiple facilitating and inhibiting effects of VP (Fig. 4) is based on the possibility of measuring compartmental release patterns of the neuropeptide within the brain. It further confirms the evolutionary advantage of involving VP in a regulatory pattern: The compartmental and site-specific release makes it possible to involve the same neuropeptide for differentiated and partially opposing effects, which seems to enable an economical and extremely fine-tuned regulation. Accordingly, whenever possible, attempts to study the physiological contribution of centrally-released VP to endocrine, autonomic, immunological and behavioral regulation have to use 'direct' (i.e., site-specific) rather than 'global' (e.g., intracerebroventricular) approaches for tasks such as sample collection and substance administration.

The finding that the same neuropeptide, namely VP, may influence the activity of the HPA axis in completely different ways not only raises questions as to the fine-tuned coordination of these regulatory patterns but also provides the opportunity to interpret alterations of this axis observed in psychiatric disorders (Holsboer, 1995) under these novel points of view.

VP – A 'psychoneuro-immunological' neuropeptide

The neuroendocrine and immune systems are closely interrelated, and endogenous VP is likely to play a critical role in this dialogue. Thus, our first question was whether the cytokine interleukin-1β known to be synthesized and released in the brain is capable of altering the central release of VP. Because both the SON and PVN receive an interleukin-1β innervation (Lechan et al., 1990) and are therefore likely to act as interfaces in the coordination of neuroendocrine, autonomic, immunological and behavioral effects, neuropeptide release was measured in these nuclei and, additionally, into blood. After intracerebroventricular administration in urethane-anesthetized rats, the interleukin-1β-induced release into blood was accompanied by changed intra-SON release patterns, giving rise to the hypothesis that VP (and OT) are putative candidates for physiological modulators of cytokine actions at the brain level. Following interleukin-1β infusion directly into the respective nucleus, VP release was increased in the SON and tended to be increased in the PVN (Landgraf et al., 1995c). The latter finding is in contrast to that of Watanobe and Takebe (1993) who succeeded in detecting a significant rise in the PVN using the push-pull perfusion technique. It remains to be shown whether the marked response of VP release demonstrated in their study is due to the different experimental conditions, including the relatively high degree of damage to the PVN area necessarily caused by the push-pull perfusion procedure. Whatever the reason for this discrepancy may be, the results suggest that VP release within the hypothalamic nuclei may differentially respond to a given stimulus, in this particular case to interleukin-1β.

Given that intrahypothalamic release of VP reflects a more general activation of the neuropeptide in the rat brain as has been demonstrated by Engelmann et al. (1994) interleukin-1β, by stimu-

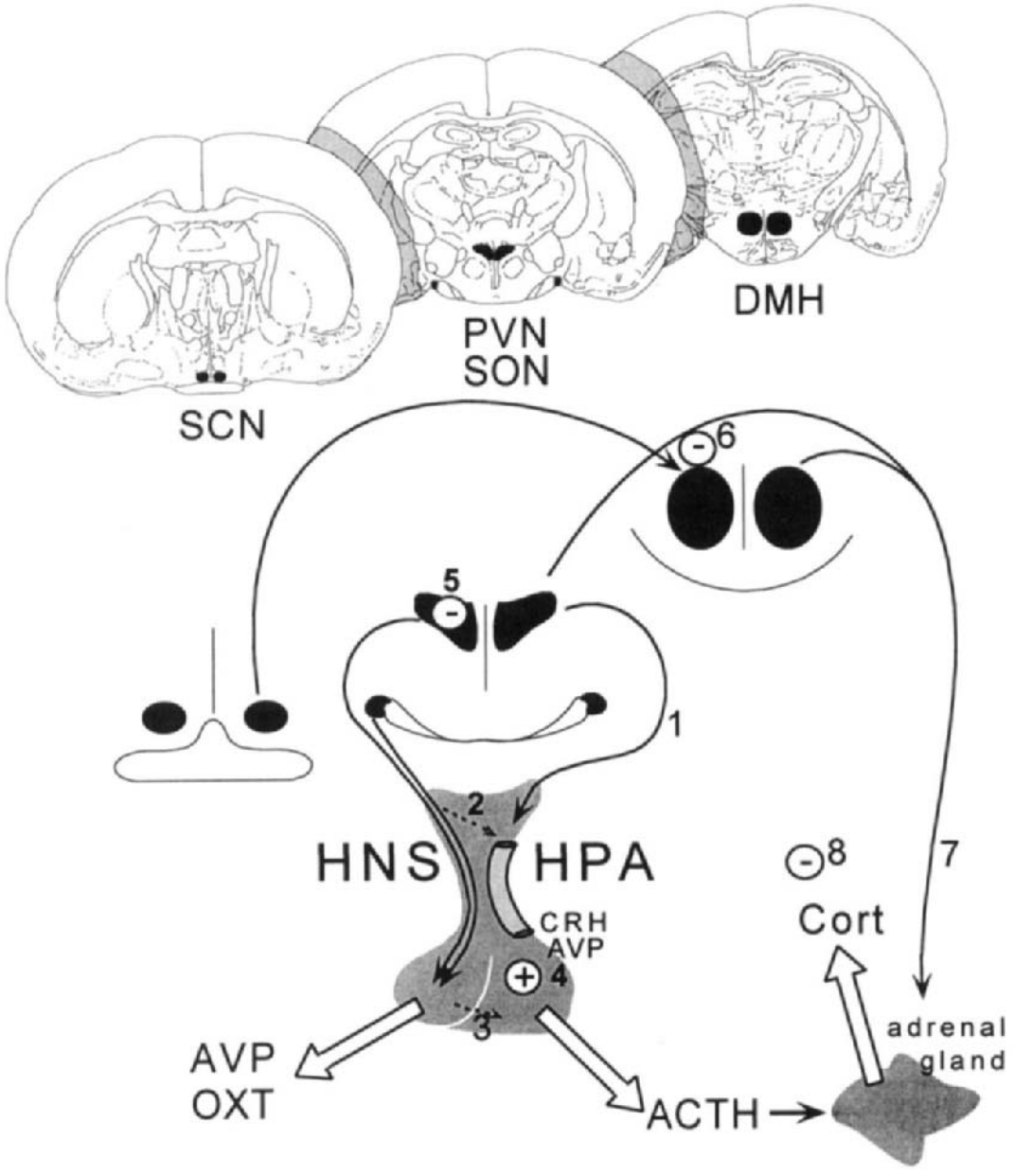

Fig. 4. Attempt to illustrate the physiological involvement of endogenous VP in HPA axis regulation of the rat. (1) Released from parvocellular neurons of the PVN into the external zone of the median eminence, VP is transported by portal blood to the adenohypophysis. (2) Released en passant from axons of magnocellular neurons running through the internal zone of the median eminence to the posterior pituitary, VP is thought to gain access to the portal blood. (3) VP could reach the adenohypophysis via short portal vessels after its release from nerve terminals in the posterior pituitary. (4) CRH and VP synergistically stimulate ACTH secretion from the adenohypophysis. (5) Originated and released within the PVN, VP inhibits ACTH secretion by as yet unknown mechanisms. (6) Originated in and transported from the SCN to the DMH/PVN area, VP inhibits corticosterone secretion from the adrenal gland probably by influencing DMH/PVN neurons with descending projections to brainstem and spinal cord (see 7). (7) Sympathetic innervation of the adrenal gland. (8) Negative feedback action of corticosterone on HPA axis activity.

lating the central release of VP, might induce the release of its opponent, thereby limiting the actions of the pyrogen to cause fever and sickness behavior. Although the central release of VP in response to interleukin-1β does not seem to be primarily due to the rise in body temperature (Landgraf et al., 1995c), it could act to oppose the central effects of the cytokine on fever (Pittman et al., 1988; Landgraf et al., 1990b) and sickness behavior (Bluthé and Dantzer, 1992). In this context it is noteworthy that central administration of interleukin-1β has been shown to reduce anxiety-related behavior (Montkowski et al., 1997), an effect opposite to that induced by VP (Liebsch et al., 1996). Brain areas predominantly involved in these processes are the septum (Pittman et al., 1988; Landgraf et al., 1990b; Liebsch et al., 1996) and the PVN (Horn et al., 1994). Hence, the physiological significance of endogenous VP might be to mitigate the undesirable effects of unopposed cytokine actions that otherwise are likely to disturb homeostasis and to be harmful to the organism. Accordingly, microdialysis stimulation of the SON with hypertonic sodium caused a reduced fever in febrile rats (Landgraf et al., 1994), a finding which, although not definite proof, provides further support for the hypothesis of an involvement of central VP in antipyresis.

The low concentration of VP in plasma, even in response to interleukin-1β, makes it uncertain whether the variety of peripheral effects of the neuropeptide on immune functions, including those on B- and T-cell proliferation, interleukin secretion etc. (Enzmann et al., 1996), are physiologically relevant. But even if not, the well-described actions of plasma VP on antidiuresis might contribute to the body's homeostatic responses to cytokines induced by infection. In this way, plasma VP could indirectly support the multitude of central effects of centrally-released VP. It has to be shown whether or not this hypothetical synergistic action of central and peripheral VP is typical after coordinated release patterns.

Several lines of evidence confirm the interaction between interleukins and VP. Interleukin-2, for example, has been shown to increase the expression of the VP precursor gene in the hypothalamus of nude mice (Pardy et al., 1993) and VP shows close linkage with interleukin-1 on mouse chromosome 2 (Marini et al., 1993).

Conclusions

As supported by the approaches used so far, the spatial and temporal pattern of VP release within the rat brain is considerably more differentiated than initially believed. Dependent on the kind and intensity of stimulation, this central release can be accompanied by peripheral release from the posterior pituitary. Furthermore, it can be determined by a variety of stressors, including emotional stressors such as social defeat, which typically stimulate central, but not peripheral VP release. More than for OT, this differentiated release into both compartments seems to be a feature of VP. Future studies will have to focus on this striking difference between VP and OT, as well as on possible interactions between central and peripheral release patterns. After its central release and subsequent neuropeptide-receptor interactions, VP is involved in the regulation of emotional and cognitive components essential for adequate behaviors and for the development of stress-coping strategies. As the release of VP is closely linked to emotionality/learning/memory, neuroendocrine, autonomic and immunological functions, investigations into the regulation of VP release within the brain and its physiological and behavioral consequences may provide some insight into a possible mechanism for the evolution of central neuropeptide release patterns and behavioral performance. It should, however, not be ignored that all the methods used so far to characterize the interaction between central neuropeptide release and the functions mentioned above have their pitfalls. Most of the approaches, for instance, are limited by their correlative nature. Also, the critical link between central VP release, emotionality/learning/memory and behavioral performance may not be 'observed' easily given that these events may not necessarily occur simultaneously and may even be separated by significant and variable delays. Thus, in view of the breath-taking progress of VP-related research, only one thing seems to be absolutely certain - namely that even the extended concept

of central release and effects of VP discussed in this article will have to be updated in just a few years.

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CHAPTER 3.1.5

Vasopressin and sensory circumventricular organs

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The subfornical organ, the area postrema and the organum vasculosum of the lamina terminalis are considered to be sensory circumventricular organs as they contain neuronal somata which are located outside the blood–brain barrier and are thus capable of serving as 'sensors' for blood-borne humoral messengers. The endocrine hormone, vasopresssin (VP), not only causes strong antidiuresis by acting on the kidney, but also exerts centrally mediated effects as a neuromodulator. Several lines of evidence suggest that VP can influence regulatory functions mediated by the sensory circumventricular organs, since vasopresssinergic somata and terminals as well as VP receptors have been reposted to be present in these structures. These biochemical prerequisites offer the possibility that blood-borne VP might on the one hand act as a feedback signal from the periphery and, on the other hand, synap-tically released or locally produced VP could modulate the known functions of sensory circumventricular organs, such as thirst, fever or cardiovascular regulation. This review focuses on the possible physiological relevance of VP acting on sensory circumventricular organs in view of recent evidence obtained from biochemical and electrophysiological studies at the cellular level.

Introduction

This chapter focuses on the role of arginine[8]-vasopresssin (VP) in the specialized brain regions known as the circumventricular organs (CVOs). Although the physiological importance of circumventricular organs as vital structures involved in the maintenance of various homeostatic functions has been well studied, their small anatomical size has hampered a thorough investigation of their biochemical character. Characteristic features of circumventricular organs are their location on ventricular walls of the brain and the lack of a blood–brain barrier, which endows these structures with the potential to monitor peripheral hormonal stimuli. The circumventricular organs located in the rostral wall of the third cerebral ventricle include the subfornical organ and the organum vasculosum of the lamina terminalis (OVLT). These brain nuclei, together with the area postrema (AP), a circumventricular organ found in the hindbrain adjacent to the fourth ventricle, are considered primarily as sensory structures. They can be distinguished by the presence of neuronal perikarya, which are not evident in other CVOs. The best investigated functions of sensory CVOs are their contributions to the control of body fluid balance and cardiovascular homeostasis. As such, they are intimately involved in the physiological responses governed by VP. Released from the neurohypophysis into the circulation, VP acts as an antidiuretic and natriuretic factor in the defense of appropriate body fluid balance. Stimulation of the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis leads to the release of VP from the neurohypophysis. In addition, VP can influence the cardiovascular control mechanisms mediated by the AP. The widespread system of brain-intrinsic vasopressinergic neurons is assumed to modulate autonomic reactions associated with fever and particular components of behavior and memory. Central VP acts as an antipyretic in fever caused

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by exogenous pyrogens which may affect thermoregulatory centers via the SFO or the OVLT, both closely coupled with the VP-synthesizing neurons of the supraoptic nucleus. Recent findings indicate that the sensory CVOs express various subtypes of VP receptors, are innervated by vasopressinergic projections and contain VP-mRNA. Therefore, these structures possess a complete biochemical system to sense, produce and respond to VP. To date, the physiological consequences of these findings obtained at the cellular level remain to be identified.

Circumventricular organs as 'windows to the brain'

Up to 18 different brain structures have been classified as CVOs within the vertebrate phylum. In most mammalian species, however, only eight brain structures considered as CVOs are typically found in the brain. They include, in higher vertebrates, the aforementioned sensory structures OVLT, SFO and AP, as well as the median eminence, the intermediate and neural lobes of the pituitary, the subcommissural organ and the pineal gland. The CVOs have characteristic features which distinguish them from other brain structures, most notably the mid-line location along the ventricular walls of the brain (Fig. 1) and (with the exception of the subcommissural organ) the lack of a blood–brain barrier (BBB).

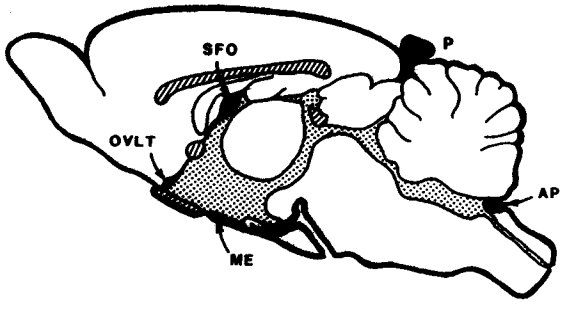

Fig. 1. Schematic drawing of the CVOs in the mid-sagittal section of the rat brain. OVLT, organum vasculosum of the lamina terminalis; SFO, subfornical organ; ME, median eminence; AP, area postrema; P, pineal gland.

Although their proximity to the ventricular system led to their name, the lack of a BBB largely defines CVO functions. Systemic administration of vital dyes has allowed anatomists to observe the morphological basis for the BBB deficiency (Leonhardt, 1980). The CVOs are remarkable in the fenestration of their capillary endothelium, their extensive vascularization and large perivascular spaces (Spoerri, 1963; Gross, 1987, 1991; Dellmann, 1985). Their capillary endothelium and specialized ependyma show extensive pinocytotic activity. Although tight junctions between the ependymal cells prevent passive diffusion of substances into the CVOs from the cerebrospinal fluid (CSF), specialized cells, called tanycytes, are believed to mediate the transport of selected substances between the CSF and perivascular spaces. Based on these unique morphological features, researchers have suggested that CVOs may serve as interfaces between the brain, the circulation and the CSF allowing for integration of neural and circulating signals (Gross, 1987; McKinley et al., 1990; McKinley et al., 1996; Johnson and Gross, 1993). This proposed communicative role of the CVOs led to the synonym 'windows to the brain'. The median eminence, the pituitary lobes and the pineal gland are generally considered as secretory structures, in contrast to the proposed sensory function of the SFO, OVLT and AP (Johnson et al., 1992; Johnson and Gross, 1993). Within the CVOs, the presence of neuronal cell bodies which project to regions lying within the BBB is considered to be a morphological prerequisite for such sensory function. This notion is further supported by the presence of various receptors for steroids, monoamines, amino acids and for peptides, including receptors for angiotensin II (AngII) and arginine VP (Johnson and Gross, 1992; Palkovits, 1987). A corresponding list of neurotransmitters and hormones have been identified in the CVOs by immunohistochemistry and biochemical extraction suggesting that they could also serve an additional endocrine function (Landas and Phillips, 1987; Summy-Long, 1987). Although it cannot be excluded that CVOs accumulate these substances from the circulation, there is growing evidence that the CVOs may synthesize them directly, and/or may receive them from efferent neuronal projections.

The importance of these small BBB-free structures is underscored by the list of proposed functions in which they are thought to participate: maintenance of body fluid homeostasis, blood pressure-, temperature-, and respiratory regulation, energy balance, immune response, pain modulation, emesis, taste aversion, biological rhythms, reproduction, parental behaviors, lactation, growth, sleep, arousal, and attention (for review, see Gross, 1987; Johnson and Gross, 1993).

Together, the specialized cytoarchitecture, the strategic location of the CVOs on ventricular walls, the lack of a BBB and the bidirectional neuronal connections with the brain parenchyma give sensory CVOs the potential to sense substances in the circulation and the CSF. In addition, they can be modulated by neuronal projections and/or convey information to the brain (Fig. 2).

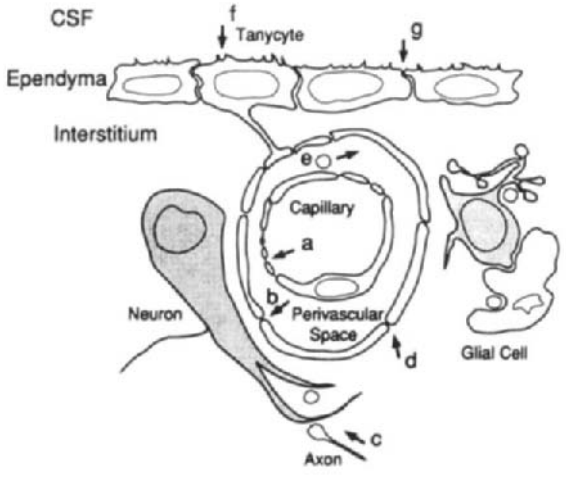

Fig. 2. Schematic diagram of sensory CVO microanatomy that demonstrates how VP may reach CVO neurons. Plasma-borne VP passes fenestrated capillaries into large perivascular spaces (a) and interstitium (b), where it can influence neurons and glial cells. Neurons are interconnected to efferent projections (c). VP produced within the CVOs can leak from the interstitium into perivascular space (d), where also axonal nerve terminals are found (e). VP from the CSF may reach the interstitium and perivascular space via active transport through tanycytes (f). Passive diffusion from the CSF (g) is excluded by tight ependymal junctions (modified from Pickel and Armstrong, 1984).

VP and homeostatic functions of the sensory CVOs

In order to maintain appropriate osmolality of the extracellular fluid, terrestrial animals have developed behavioral mechanisms such as thirst and sodium appetite as well as physiological functions controlling the output of water and sodium. Vasopressin is an important hormone for the control of renal functions including water and sodium excretion. On the other hand, the sensory CVOs of the lamina terminalis, the OVLT and SFO have been implicated in osmosensation, control of VP release, drinking and salt appetite (Johnson et al., 1992; Bourque et al., 1994; Thunhorst and Fitts, 1994; Richard and Bourque, 1995). Together, these findings implicate a very tight functional coupling between VP and sensory CVOs in the control of fluid balance.

The OVLT is located in the most ventral part of the lamina terminalis, along the rostral wall of the third cerebral ventricle (McKinley et al., 1987). It is bidirectionally interconnected with the VP-synthesizing magnocellular neurons of the supraoptic (SON) and paraventricular nucleus (PVN), the median preoptic nucleus (MePO) and the SFO (Phillips and Camacho, 1987). Experiments have established the functional importance of the OVLT as a major osmoreceptive site involved in the control of the activity of SON neurons resulting in VP release (Richard and Bourque, 1995; Bourque et al., 1994). Because of the exceptionally small size of the OVLT (150–300 μm in the rat) (Landas and Phillips, 1987) it has often been anatomically included to the region surrounding the anteroventral third ventricle (AV3V region), particularly when investigated with techniques having limited spatial resolution such as lesion studies. However, such lesion studies have established the importance of the OVLT, SFO and the interconnected MePO in AngII- and osmotically induced drinking and VP secretion (Simpson and Routtenberg, 1973; Bealer et al., 1979; Thrasher et al., 1982a,b; Johnson et al., 1992; McKinley et al., 1996).

The osmoregulatory centers of the hypothalamus are reciprocally linked to the cardiovascular centers of the brain stem and an additional sensory CVO, the AP. Together with the nucleus of the solitary

tract (NTS) which receives vagal inputs from low and high pressure receptors of the circulation, from arterial chemoreceptors and possibly also from visceral (hepatic) osmoreceptors, the AP and NTS constitute the main viscero-sensory input channels of the medulla oblongata.

While early studies have focused mainly on the function of the AP as the chemosensory trigger zone for the emetic reflex (Borison et al., 1984; Carpenter et al., 1988; Borison, 1989), more recent reviews have emphasized the role of the AP in the regulation of cardiovascular function (Cox et al., 1990; Williams et al., 1992; Bishop and Hay, 1993). The AP receives afferent input from several peripheral and central cardiovascular regulatory systems including the PVN and dorsomedial nuclei of the hypothalamus, the carotid sinus and the vagus nerves. Neurons in the AP project predominantly to the NTS, the lateral parabrachial nucleus, the dorsal motor nucleus of the vagus, the nucleus ambiguous and the ventromedial medulla (Miselis et al., 1987a,b). These anatomical relationships, along with the sensory capacity of the AP for circulating substances suggest that it may act as a monitor of peripheral and central signals related to cardiovascular functions (Bishop and Hay, 1993).

The AP may be influenced by VP circulating in plasma, coming from the CSF of the adjacent 4th ventricle, from vasopressinergic fibers likely originating from the PVN (Miselis et al., 1987a; Palkovits, 1987) or from endogenous production within the AP itself (Lepetit et al., 1993). This holds true for other sensory CVOs as well. Biochemical studies have demonstrated the presence of VP in tissue extracts from the SFO and in tanycytes (Summy-Long, 1987) as well as a vasopressinergic innervation in SFO tissue slice (Weindel and Sofroniew, 1976; Sofroniew, 1985; Palkovits, 1987). Very recent findings of VP mRNA in the SFO (Lepetit et al., 1993), immunopositive fibers and cell bodies in SFO tissue slices from several species (Duann et al., 1995; Alm et al., 1997), together with the positive VP/neurophysinII (NPII) immunoreactivity observed in the SFO culture (Jurzak et al., 1995a) clearly demonstrate an endogenous source of VP within the SFO. As for the SFO, immunohistochemical evidence for vasopressinergic fibers and cells exists for the OVLT (Palkovits, 1987; Zeisberger and Merker, 1992). The vasopressinergic input to the OVLT is believed to originate from the SCN, the circadian pacemaker of the brain (Buijs, 1978). Together, the data indicate that VP may access the sensory CVOs via plasma, the CSF, from afferent projections or from endogenous production within the structures itself.

VP fragmentation

The characterization of VP function within the CVOs may be complicated by the active metabolic N- and C- terminal processing of neurohypophyseal hormones in the brain. This proteolytic activity yields fragment peptides with cleaved ring structures and leads to an accumulation of fragments with a pyroglutamate residue at the N-terminus such as the VP fragment [pGlu^4,Cyt^6]AVP 4–9 (VP 4–9) (Burbach et al., 1983; Burbach, 1986; Stark et al., 1989). Further cleavage of the C-terminal [Gly-NH_2] generates [pGlu^4,Cyt^6]AVP 4–8 (VP 4–8), another naturally occurring peptide. These VP fragments are thought to be more potent than the parental VP in memory-associated neuromodulatory functions (Burbach et al., 1983; De Wied et al., 1987, 1991), the functional tolerance to ethanol (Szabo et al., 1991) and the modulation of melatonin synthesis (Liu and Burbach, 1988; Stehle et al., 1991). Autoradiographic studies with ^{35}S-labeled VP 4–9 have revealed central binding sites different from those of the parent peptide (De Kloet et al., 1985; Brinton et al., 1986). Interestingly, a high density of [^{35}S]VP 4–9 binding sites has also been reported in CVOs (Jurzak et al., 1993, 1995c). Therefore, a comprehensive evaluation of VP function in CVOs must consider not only the effects of VP acting on the VP receptor, but also the action of its cleavage products via specific VP fragment receptors.

Heterogeneity of central VP receptor subtypes

There is growing evidence supporting the notion of a heterogeneity of central VP receptors. Most binding studies have indicated a prevalence of V_1 like receptors in the CNS (Phillips et al., 1988, 1990; Gerstberger and Fahrenholz, 1989; Tribollet et al., 1992; Barberis et al., 1995). However, more sensitive and specific receptor

mapping has become feasible with the recent sequencing of VP receptors from different species (Birnbaumer et al., 1992; Lolait et al., 1992; Morel et al., 1992; De Keyser et al., 1994; Hirasawa et al., 1994c; Thibonnier et al., 1994; Lolait et al., 1995; Saito et al., 1995). First in situ hybridization experiments with V_1 receptor specific mRNA provided evidence of V_{1a} receptor expression in the rat brain (Ostrowski et al., 1992). However, more recent data support the idea that central VP receptors may also differ from the 'classical' V_{1a} receptor at different stages of development, or in distinct brain regions. Recently, a transient expression of V_2 receptor mRNA was shown in the rat brain (Hirasawa et al., 1994a,b,c). In a more detailed study, V_2 receptor message was localized in the vascular endothelium, endothelial cells of the choroid plexus, neurons in the hippocampus and in the granular layer of the cerebellum (Kato et al., 1995). In addition, sensitive RT-PCR analysis demonstrated V_{1b} receptor expression in the rat brain as well (Burbach et al., 1995; Saito et al., 1995). A rat V_{1a} receptor probe (TM 1–5), used to screen both the rat and human genome was found to hybridize with 5–9 different bands at moderate stringency (Morel et al., 1992) supporting the notion of a VP receptor heterogeneity. In addition, alternate splicing of the V_{1a} receptor transcript has been suggested to generate pharmacologically and functionally distinct receptor variants (Lolait et al., 1995).

Biochemical evidence for VP receptors in the sensory CVOs

Autoradiographic studies in rat brain with either [^{3}H]VP or iodinated V_1 receptor specific antagonists have not shown consistent labeling in sensory CVOs. However, several studies have reported positive [^{3}H]VP-labeling of the AP and SFO (Tribollet et al., 1988; Gerstberger and Fahrenholz, 1989; Fahrenholz et al., 1993). It is noteworthy, that VP labeling in the SFO appears to be restricted to the periphery of the structure, whereas the main body remains unlabeled (Tribollet et al., 1988; Gerstberger and Fahrenholz, 1989). However, endogenous VP has little selectivity for receptor subtypes and binds with equally high affinity to VP- and oxytocin- (OT) receptors (Burbach et al., 1995). In studies performed with the V_1 receptor antagonist, [^{125}I]d(CH_2)^5,sarcosine^7-VP, specific CVO labeling was only found in the AP (Phillips et al., 1988; Gerstberger and Fahrenholz, 1989). Recently, a positive labeling of CVO structures (SFO, AP) was confirmed with the high affinity, linear V_1 receptor antagonist, [^{125}I]HO-LVA (Barberis et al., 1995). Direct autoradiographic evidence of VP receptors in the OVLT is not available. However, VP receptor labeling in the surrounding AV3V region was found (Gerstberger and Fahrenholz, 1989; Fahrenholz et al., 1993; Barberis et al., 1995; Jurzak et al., 1995a). The small size of the OVLT (150 × 300 μm in the rat) may have obscured a clear specific labeling given the limited spatial resolution of autoradiographic techniques.

The superior resolution of immunohistochemical techniques has been successfully employed to reveal extensive VP receptor labeling in the CVOs (Jurzak et al., 1993). Since purified receptor antigen or synthetic peptides were not available at the time of the study, antibodies derived from an anti-idiotypic route were employed. In this study, an idiotypic VP-binding antibody was used as a substitute antigen for the ligand binding site in order to generate anti-idiotypic- and thus, receptor cross-reactive-antibodies (Strosberg, 1987; Linthicum and Farid, 1988; Wang, 1990). This anti-idiotypic antibody was shown to label VP receptors in both, peripheral liver and kidney tissue as well as on cell lines expressing V_1 and V_2 receptors, (Jurzak et al., 1992, 1993; Fahrenholz et al., 1993). In rat brain, positive anti-idiotypic antigen staining has been obtained in the sensory CVOs (AP, SFO, OVLT) as well as in the pineal gland, median eminence, posterior pituitary and choroid plexus. In addition, non-CVO nuclei such as the arcuate nucleus and NTS have been labeled, two structures having extensive neural connections with the CVOs and likewise reported to have a leaky BBB (Gross et al., 1990; Shaver et al., 1992). Furthermore, the SON, the inferior olive and the ventricular ependyma were also labeled by the antibody. This staining pattern does not

correspond with findings obtained by receptor autoradiography with VP receptor ligands or in situ hybridization using cloned receptor probes. This staining pattern did, however, show a strong correspondence to that obtained from autoradiographic studies using [^{35}S]VP 4–9, the major VP-metabolite (Jurzak et al., 1993). Early studies indicated a unique binding site distribution of the VP fragment in the brain, distinct from that of the VP receptor (Brinton et al., 1984, 1986; De Kloet et al., 1985). The pattern of the anti-idiotypic antibody labeling and the specificity of the idiotypic antigen used to derive the antibody (Jurzak et al., 1990) strongly suggest that this antibody recognizes a VP fragment receptor in the CVOs.

In our hands the labeling of CVOs and neighboring structures with radiolabeled [^{35}S]VP 4–9 was by far the most intense and prominent labeling in the rat brain. Others report stronger labeling in the hippocampal and cortical regions using either [^{35}S]VP 4–9 (De Kloet et al., 1985; Brinton et al., 1986) or the shorter [^{35}S]VP 4–8 fragment (Du et al., 1994a,b). Based on the labeling pattern in the hippocampal pyramidal cell layer and granular cell layer of the dentate gyrus, the latter study speaks in favor of a separate binding site for both VP fragments. A further investigation of [^{35}S]VP 4–8 fragment binding on rat cortical synaptosomal membranes suggested again that the VP fragment receptor is pharmacologically distinct from VP receptors. However, the authors did not address the possible subdivision of VP fragment receptors into VP 4–8 and VP 4–9 receptors (Du et al., 1994a,b). Central VP receptor membrane preparations have been shown to be particularly sensitive to differences in concentration of ions such as Mg^{2+}, Ni^{+} and Zn^{2+} in the buffer solutions (Barberis et al., 1992; Junig et al., 1985). Therefore, a direct comparison of studies using different protocols may be misleading. Particularly in the light of the recent discovery of a ligand-induced cleavage of VP receptors by a Zn^{2+}-sensitive metalloproteinase (Kojro and Fahrenholz, 1995), it is evident that small variations in buffer composition may lead to profound changes in the receptor pharmacology and staining patterns in autoradiographic studies.

For the investigation of CVO regions, where the amount of available tissue precludes a biochemical approach for VP receptor studies, other techniques have to be employed. Therefore, a few groups have attempted to use primary cell cultures as tools to study VP receptor function and pharmacology on single cells, thereby avoiding the problems of limited tissue availability and proteolytic activity in broken membrane preparations.

CVO cell culture

The proposed heterogeneity of central VP receptors has gained strong support from studies performed in CVO cell culture. Single cell Ca^{2+}-measurements in primary cell culture derived from the OVLT, SFO and AP have been performed (Hay et al., 1993; Jurzak et al., 1994, 1995b; Consolim-Colombo et al., 1996) using fura2-loaded cells. In addition to allowing the study of VP receptor pharmacology, such cell culture preparations can be used to classify the responding cell types by their characteristic morphology and/or reactivity with cell type-specific antibodies. Calcium responses to VP were found in the OVLT and SFO culture in neurons and astrocytes (Jurzak et al., 1995b). The morphological distinction of the cell types was confirmed by cell type-specific antibodies, neuronal specific enolase and glial fibrillary acidic protein, respectively. Among cultured neurons, 34, 28 and 38 % of SFO, OVLT and AP cells, respectively, responded with an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) after addition of VP (Table 1). Interestingly, whenever tested, a comparable portion of astrocytes showed VP-induced $[Ca^{2+}]_i$ transients as well, with 62 % of SFO and 38% of OVLT astrocytes responding. In neurons and astrocytes the responses persisted even when extracellular calcium was omitted from the medium, indicating that the Ca^{2+}signal was derived from intracellular sources. The V_{1a} specific antagonist, $d(CH_2)_5[Tyr(Me)^2]$-VP, (Manning-Compound) blocked VP responses in all CVOs and in both cell types tested (Fig. 3). The release of Ca^{2+} from intracellular stores and the indicated pharmacology speak in favor of a V_{1a} receptor subtype in the CVO cultures. However, a significant number of neurons (8 of 18 tested) in the OVLT culture responded with a $[Ca^{2+}]_i$ transient after application of dDAVP, a selective V_2 receptor agonist, suggest-

ing that an additional VP receptor subtype was present. Only one dDAVP-induced Ca^{2+} transient was observed in cultured SFO neurons and none of the tested astrocytes were dDAVP-sensitive. These findings indicate that a VP receptor, sharing the pharmacology of a V_2 receptor and the signal transduction system of the V_1 subtype, is preferentially expressed in neurons of the OVLT culture. In addition to dDAVP-sensitive receptors, Ca^{2+} responses to OT, the other neurohypophyseal hormone, were also observed in a significant number of neurons and astrocytes in SFO and OVLT culture (Table 1).

In addition to OT receptors, the SFO and OVLT cultures have also provided evidence for a functional VP fragment receptor in neurons and astrocytes of these CVOs (Jurzak et al., 1995c). A small, but consistent percentage of SFO and OVLT neurons and astrocytes responded to application of the VP 4–9 fragment peptide with a $[Ca^{2+}]_i$ transient. In contrast to VP-evoked signals, all VP 4–9 responses were characterized by a strong tachyphylaxis, thereby complicating the investigation of receptor pharmacology. However, 4 of 6 VP 4–9-sensitive neurons elevated $[Ca^{2+}]_i$ after application of the shorter VP 4–8 fragment as well, indicating that both VP fragments may stimulate the same receptor. The notion of a common receptor for the VP 4–8- and VP 4–9 fragments was supported by the finding that both VP fragments could compete with [^{35}S]VP 4–9 for binding sites in the autoradiographic labeling of CVOs, whereas the parent VP and the analogous avian hormone, vasotocin (AVT) did not (Jurzak et al., 1995c). Very recent observations indicate a similar lack of competition by parent VP/AVT for [^{35}S]VP 4–9 binding in other species, indicating the presence of separate VP fragment receptors in other vertebrates as well (Hübschle et al., in preparation).

That only a relatively low percentage (7–8%) of cultured CVO cells showed a functional response to the VP fragments appears to be in contrast to the strong autoradiographic VP fragment labeling of the CVOs in the tissue slice. Since only measurements of $[Ca^{2+}]_i$ were employed, activation of other messenger systems might not have been detected. However, reports showing VP fragment induced inositol phosphate mobilization, support the notion that the VP fragment receptor subtypes might indeed be coupled to the phospholipase C and $[Ca^{2+}]_i$ signal transduction cascade (Gu and Du, 1992).

These results indicate that VP-evoked responses within the CVOs are likely mediated by neurons and astrocytes which are activated by several related, but distinct (V_{1a}, VP fragment- and dDAVP sensitive) receptors.

Table 1
Summary of Ca^{2+} transients obtained in single cultured astrocytes and neurons isolated from the OVLT and SFO (Jurzak et al., 1995a,b) and AP[a]

Substance	OVLT[b]		SFO		AP	
	Neurons	Astrocytes	Neurons	Astrocytes	Neurons	Astrocytes
VP	26 (92)	13 (34)	19 (56)	21 (34)	6 (6)[c] 63 (164)[d]	n.t n.t.
V_{1a} antagonism[e]	9 (9)	5 (5)	3 (3)	6 (6)	15 (15)[d]	n.t.
dDAVP	8 (18)	0 (9)	1 (14)	0 (17)	n.t.	n.t.
OT	10 (31)	17 (27)	7 (13)	14 (33)	n.t.	n.t.
VP 4–9	8 (102)	2 (26)	4 (53)	2 (27)	n.t.	n.t.

[a] The number of responsive cells is given with the number of tested cells in parenthesis. n.t. = not tested.
[b] Due to the small size of the OVLT, the culture must be considered as OVLT/AV3V culture.
[c] Hay et al. (1993).
[d] Consolim-Colombo et al. (1996).
[e] Inhibition of the VP response by the Manning Compound.

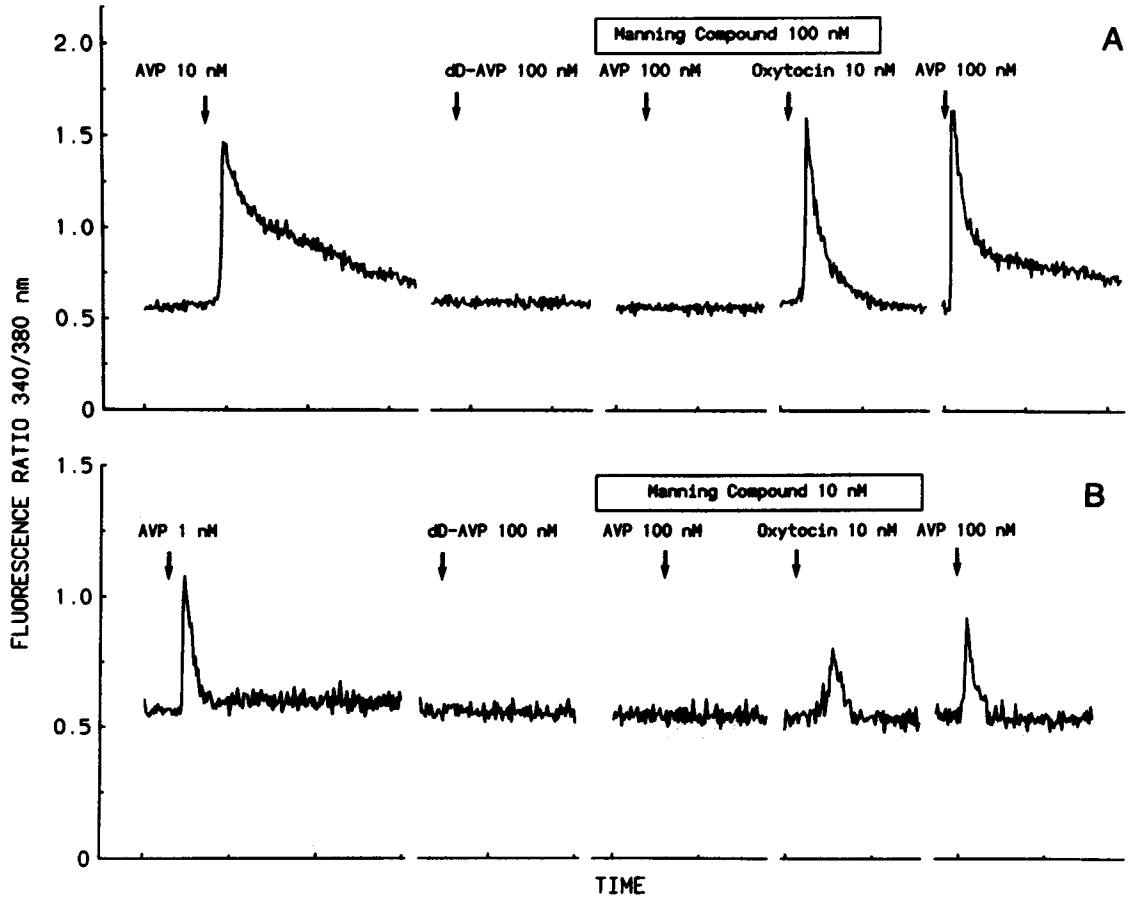

Fig. 3. Examples of the characterization of Ca^{2+} responses in cultured CVO cells. The tracing in A was obtained from a single SFO astrocyte, which responded to VP but not to dDAVP. The response to VP, but not to OT, was blocked by the V_{1a} receptor specific antagonist Manning Compound. Washout of the antagonist led to restoration of the responsiveness to VP. The tracing in B was obtained in a single OVLT neuron and shows an identical response pattern indicating the same receptor pharmacology. Time between ticks represents 100 s (according to Jurzak et al., 1995a).

Astrocyte plasticity and morphology of the sensory CVOs

The VP-sensitive CVO neurons are thought to convey information via neural projections to higher centers within the BBB and/or to act as interneurons to modify the activity of other systems within the CVOs. The role of astrocytes, however, is less well understood. Aside from the well recognized glial cell functions such as potassium and neurotransmitter uptake and inactivation of extracellular peptide levels by endopeptidase activity (leading to VP fragmentation?), the specialized cytoarchitecture of the CVOs suggests that astrocytes might have here additional functions. Most of the data relating to the role of astrocytes in CVOs have been obtained from the neurohypophysis. Morphological plasticity of the neurohypophyseal pituicytes, in vivo and in culture has been demonstrated and attributed to altered ionic buffering, state of neuronal activity and stimulus-secretion coupling (Bicknell et al., 1989; Beagley and Hatton, 1992; Hatton, 1997). Pituicytes have been shown to change their morphology during osmotic

challenge (hypertonic saline or dehydration) and β-adrenergic stimulation (Bicknell et al., 1989; Beagley and Hatton, 1992). In addition, pituicytes were found to proliferate in salt-loaded animals (Murugaiyan and Salm, 1995) and after exposure to VP and OT (Lucas and Salm, 1995). Interestingly, salt treatment increased the cross-sectional area of the vascular portion of the posterior pituitary (Livingston, 1973), further underscoring the tight functional coupling between astrocytes and endothelial cells, which is believed to be the cellular basis for the maintenance and regulation of the BBB (Janzer, 1993). Pituicytes, as specialized astrocytes of the neurohypophysis, demonstrate a morphological plasticity which might be relevant for other CVOs as well. This hypothesis is supported by observations of morphological changes of SFO astrocytes in primary culture (Ramsell and Cobbett, 1997; and own observation). It is tempting to speculate that astrocytes in sensory CVOs might change their morphology in response to certain stimuli (salt, VP, VP fragments or other) and thereby influence the morphology of the perivascular space and thus the perfusion rate within the CVOs.

It should be noted that despite their small size, the sensory CVOs are not homogeneous structures, but rather can be subdivided into several cytoarchitectural regions according to the leakiness of their capillaries, immunoreactivity for transmitters and peptides and their receptor distribution. (Dellmann, 1985; Gross, 1992). In the SFO up to 7 different subdivisions have been described with three different types of capillaries (Gross et al., 1990; Gross, 1992). The rostral region, as well as the outer rim of the organ, contain capillaries with tight junctions and no perivascular spaces (type II capillaries) indicating the presence of a BBB. Only the central and caudal subdivisions of the SFO contain the 'CVO typical'-type I and III fenestrated capillaries distinguished by the increasing size of their perivascular spaces. This suggests the existence of diverse functional elements within these subregions. Indeed, the SFO shows c-Fos reactivity within the core and peripheral rim of the organ after hypovolemic or hypertonic stimuli, respectively (Oldfield et al., 1994). The hypovolemic c-Fos staining parallels patterns observed with AngII receptor autoradiography (Mendelsohn et al., 1984) and corresponds to the majority of leaky capillaries in the center of the SFO. In addition, serotonin has been found almost exclusively in the center of the organ surrounding perivascular spaces of leaky capillaries (Lind, 1986). In contrast, the VP and VP fragment binding sites found in the peripheral rim of the SFO coincide with the neuronal population activated by i.p. hypertonic saline (Smith and Day, 1995). In addition, it has recently been shown that cells containing nitric oxide synthase and cells responding to nitric oxide with increased cGMP levels are found in the rostral part and in the lateral rim of the organ (Rauch et al., 1997).

Like the SFO, the AP is a heterogeneous nucleus, having several subregions (Gross, 1991) which differ histologically with respect to their vascular permeability, neuronal cell density as well as receptor and neurotransmitter content. In coronal sections, dorsal, medial and ventral regions can be discriminated. The dorsal region, or mantle zone, contains various types of glial and kinocilia-free ependymal cells (Leslie et al., 1989). These cells were found to form a tight barrier against the passive entry of the tracer molecule, horseradish peroxidase, when injected into the ventricular system (Ferrario et al., 1987). Therefore, it is unlikely that molecules within the CSF, such as neuropeptides, would be able to reach the bipolar neurons located within the dorsal AP by passive diffusion (Chernicky et al., 1980; Leslie, 1986). However, the high number of vesicles found within ependymal tanycytes and their extensions into the perivascular spaces of the AP, may provide a mechanism for an active cellular exchange with the CSF. The medial zone contains the majority of afferent and efferent connections within the AP. This region also contains fenestrated capillaries with large perivascular spaces and so comprises the portion of the AP which likely acts to detect blood-borne substances. The ventral zone of the medial and caudal AP has neural and vascular contacts with the underlying NTS, whereas the more rostral region contains very few capillaries (Shaver et al., 1991, 1992) and is separated from the NTS by a barrier of glial cells. This glial cell layer is thought to function as a barrier against the rapid diffusion of blood borne substances away from receptive cells in the AP. Anatomists have early proposed such a func-

tional blood–brain barrier surrounding the CVOs (Krisch et al., 1978), which was later implemented into a functional model (Phillips and Camacho, 1987). This model suggests that a glial barrier surrounding the core of the CVOs acts to restrict the dissipation of plasma-derived substances into the brain parenchyma. A partial opening of such a CVO-brain-barrier could act to couple an additional set of sensory neurons to the circulation within the CVOs itself and in adjacent areas. Interestingly, such a partially leaky BBB has been reported for subregions of the arcuate nucleus and the NTS, two structures which neighbor the median eminence and AP, respectively (Gross et al., 1990; Shaver et al., 1992). It is still a matter of debate whether infiltration of these nuclei follows a transcapillary or low-resistance paravascular pathway through the interstitium. Fenestrated capillaries have been detected within the vasculature of these nuclei favoring the former theory. However, it is tempting to speculate that a pericapillary dispersion could be mediated by morphological changes of astrocytes in response to appropriate stimuli. Although it appears that the sensory CVOs posses the necessary cellular mechanisms to enable such a response, direct experimental evidence for this hypothesis is still lacking.

Physiological effects of VP in sensory CVOs

In order to investigate physiological functions of blood-borne hormones via the sensory CVOs, lesion studies and direct microapplications have helped to define their roles as sensory brain structures located outside the BBB. In those cases in which the specific hormone caused the same physiological response as a non-selective electrical or chemical stimulation, an excitatory effect of the hormone on neuronal activity could be assumed. A classical way to investigate cellular responses of individual neurons is to record their electrical activity in the absence and in the presence of the hormone. Only in recent years, several attempts have been made to investigate electrophysiologically the cellular basis of the effects of VP on functions mediated by the SFO and AP, while recordings from the OVLT are not available.

Subfornical organ

The appealing idea that the antidiuretic hormone which acts on the kidney to prevent water loss could also act at a central site to defend against volume loss by stimulating water-intake was hypothesized by several authors (Szczepanska-Sadowska et al., 1974, 1982; Evered, 1983). However, a dipsogenic effect of circulating VP has so far not been clearly established in mammals. As has been reviewed in previous chapters, the SFO has all the necessary anatomical and functional components to serve as an important dipsogenic target for blood-borne VP to induce drinking. It has been identified as a structure which is critical for water-intake and contains VP receptors. In addition, the SFO is involved in the control of the release of VP from the neurohypophysis, of blood pressure regulation and salt appetite. However, most thoroughly investigated is the involvement of the SFO in the stimulation of water intake in response to circulating AngII (Iovino and Steardo, 1984; Mangiapane et al., 1984; McKinley et al., 1990; Johnson et al., 1992). It is well established that blood-borne AngII, a peripheral peptide hormone produced in response to decreased blood volume, activates neurons in the SFO. Circulating AngII acts on AT_1 receptors which are present in abundance in the SFO of various species (Fitzsimons, 1979; Simon et al., 1992; Johnson and Gross, 1993).

Electrophysiological and in vivo effects of VP in the subfornical organ of mammals

Based on the fact that electrical excitation of SFO neurons, stimulates drinking (Smith et al., 1995) one can postulate that a substance which predominantly activates SFO neurons should have a facilitatory effect on water-intake. However, this correlation does not address the question, whether a cell which responds to a given substance is actually involved in water intake or might serve other functions. The ideal experiment to test such a relationship, i.e., in vivo recording of individual SFO neurons in a conscious animal, while simultaneously monitoring its water-intake, would be technically difficult, not least due to the small size of the SFO. Therefore, we and others have used an in vitro approach, which compares the effect of a substance

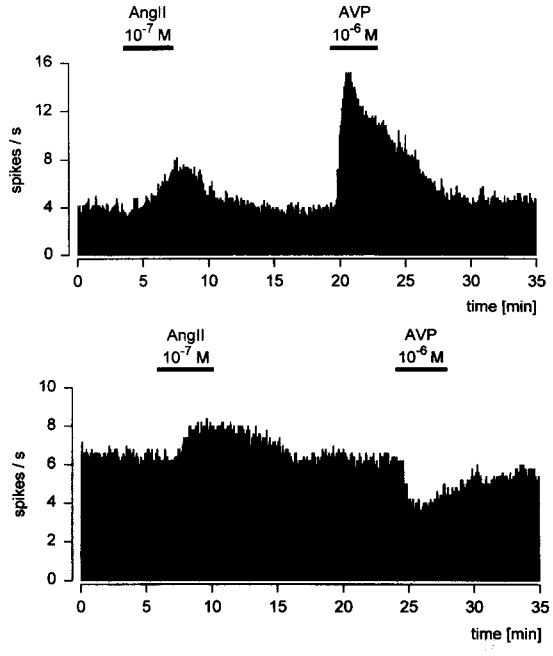

Fig. 4. Continuous extracellular rate meter recording of two spontaneously active rat SFO neurons in an in vitro slice preparation. Both cells were excited by superfusion with AngII, but VP caused an excitation in the upper and an inhibition in the lower recording (according to Anthes et al., 1997).

putatively acting on the SFO with the effect of AngII on identical SFO neurons. With this approach it has been shown that atrial natriuretic peptide (ANP) and nitric oxide (NO), both of which inhibit AngII-induced water intake (Nicolaidis and Fitzsimons, 1975; Ehrlich and Fitts, 1990), have an inhibitory effect on SFO neurons which are excited by AngII (Schmid and Simon, 1992; Rauch et al., 1997; Schmid, 1998). In contrast, calcitonin, which activates largely the same neurons in the SFO as AngII, also stimulates water-intake after peripheral application (Schmid et al., 1998). This comparative approach has also been used recently to develop ideas about possible physiological functions of VP in the SFO (Anthes et al., 1997).

Using extracellular recordings from neurons in a SFO slice preparation of rats (Anthes et al., 1997) we found that VP excited 26% and inhibited 24% of all neurons tested ($n = 159$) (Fig. 4). The remaining 50% were insensitive to VP (Table 2). Confirming previous experiments, AngII caused exclusively excitatory effects in the majority of neurons. However, no correlation could be found between cells which were excited by AngII and excited (26%) or inhibited (30%) by VP. Both, excitations and inhibitions by VP were dose-dependent and reversible. The threshold concentrations (10^{-8} to 10^{-9} M) were similar for both types of responses, whereas the threshold concentration for the excitatory effect of AngII was at least 10-fold lower. Superfusion with a medium low in Ca^{2+} and high in Mg^{2+}, which is known to block synaptic transmission in slices (Kelso and Boulant, 1982), did not affect the excitatory responses but abolished the majority of the inhibitory responses caused by VP. This suggest that the excitatory effect of VP is direct, whereas the inhibitory effect is caused by activation of local inhibitory circuits. Both excitatory and inhibitory VP-actions were blocked by a V_1 receptor antagonist. With one exception, neurons responsive to VP did not respond to the

Table 2
Numbers of rat SFO neurons responsive to AngII and VP in electrophysiological studies on tissue slices (according to Anthes et al., 1997)

Responsiveness to AngII	Responsiveness to VP			Tested
	Excitation	Inhibition	No response	
Excitation	25	29	42	96
Inhibition	0	0	0	0
No response	14	9	27	50
Number tested	39	38	69	146

V_2 receptor agonist dDAVP. These findings are in agreement with pharmacological data describing VP-binding sites in autoradiographic (Gerstberger and Fahrenholz, 1989) and calcium imaging studies in the SFO culture (Jurzak et al., 1995b).

The lack of correlation between the AngII responsiveness and the excitatory and inhibitory effects of VP on SFO neurons in rats is in line with the available in vivo data, which have so far failed to demonstrate a dipsogenic effect of circulating VP. It has been shown that high doses of VP administered systemically had no effect on water intake in normovolemic rats and decreased water-intake slightly in 24 h water-deprived rats (Rolls, 1971). In another study, infusions of VP had no effect on water intake in water-sated dogs (Eriksson et al., 1987). It was also reported that VP decreased the threshold for osmotically-induced drinking at low doses but increased the threshold at higher doses (Szczepanska-Sadowska et al., 1974, 1982). The observed heterogeneity of VP-induced responses on SFO neurons in vitro may relate to the variable blood pressure effects observed following in vivo microinjection of VP in the SFO. It has been shown repeatedly that electrical stimulation or injection of excitatory substances, like AngII, into the SFO increase blood pressure in rats (Smith et al., 1995, 1997; Ferguson and Bains, 1996). In contrast, a recent study (Smith and Ferguson, 1997) found that direct application of VP into the SFO of rats caused a decrease in blood pressure, while VP given i.c.v. had an opposite effect, which is generally in line with the data of Thornton and Nicolaidis (1993).

In summary, the currently available data so far do not provide a clear understanding of the action of VP in the rat SFO. While the direct excitatory effect of VP on SFO neurons (Anthes et al., 1997) implies similar effects of VP and ANGII on water intake and blood pressure, direct microapplication of VP in the rat SFO caused a decrease in blood pressure which contrasts ANGII mediated effects

Electrophysiological and in vivo effects of VP in the subfornical organ of ducks

It seems likely that the well known pressor effects of systemic VP observed in mammals counteracts and thus complicates the characterization of dipsogenic and other possible effects of circulating VP on the SFO (Evered, 1992). An experimental approach to investigate blood-pressure-independent central effects of peripherally applied VP in mammals is to counteract the pressure effect with coinfusions of vasodilatory substances such as isoproterenol and sodium nitroprusside (Rettig et al., 1981; Szczypaczewska et al., 1993). However these substances might have additional direct effects on SFO neurons (Schmid et al., 1995a,b; Rauch et al., 1997).

Another approach to overcome these experimental difficulties is to characterize the central effects of peripherally applied AngII and VP in ducks, an animal model, which has proven to be excellent for the study of central effects of blood-borne VP and AngII since neither peptide causes a pressor response in birds (Simon et al., 1992). The SFO receptor system for blood-borne VP and AngII appears to be quite similar in rats and ducks with regard to the leakiness of the BBB (Schmid, 1995b) and the centrally mediated effects of both peptides (Simon-Oppermann et al., 1988; Simon et al., 1992).

In order to establish the cellular effects of these peptides we first investigated the responsiveness of SFO neurons to the avian antidiuretic hormone (AVT) and mammalian VP in slices from the duck SFO. In the duck SFO slice AVT, as well as AngII, caused exclusively excitatory effects, and 88% of neurons excited by AVT were also excited by AngII (Table 3). The pharmacology of the duck AVT receptor was found to be similar to the rat VP receptor, in that mammalian VP was equipotent to AVT in exciting neurons of the duck SFO. Moreover the excitatory effect of AVT was blocked by a V_1 receptor antagonist and like for the mammalian receptor was not mimicked by the V_2 receptor agonist dDAVP (Schmid et al., 1995b).

The exclusively excitatory effect of AVT on the majority of AngII-responsive neurons led to the prediction that both peptides should stimulate SFO-mediated drinking in ducks (Schmid et al., 1995b). Intravenous infusion of AVT (120 ng/min for 1h) which was preceded and followed by dipsogenic dose of AngII (200 ng/min for 1h) significantly increased water intake in ducks at concentrations similar to those which activate neurons recorded from tissue slices of the duck

Table 3
Numbers of duck SFO neurons responsive to AngII and AVT in electrophysiological studies on tissue slices (according to Schmid et al., 1995b).

Responsiveness to angii	Responsiveness to VP			Tested
	Excitation	Inhibition	No response	
Excitation	29	0	14	43
Inhibition	0	0	0	0
No response	4	0	19	23
Number tested	33	0	33	66

SFO (Schmid and Simon, 1996). These experiments clearly show that the antidiuretic hormone is, in principle, capable of inducing SFO-mediated dipsogenic responses at least in an animal model that does not have a pressor response to the hormone.

However, it is unlikely that blood-borne AVT alone initiates SFO-mediated drinking, since the plasma concentrations needed to stimulate water intake were much higher than normally found in the circulation (Schmid and Simon, 1996). The plasma AVT concentration in ducks (5 pM) is similar to the VP concentration found in rats (3 pM). Maximal stimulation with salt loading for several months in ducks or water deprivation for 60 h in rats results in plasma concentrations of 23 pM and 15 pM, respectively (Möhring et al., 1980; Cowley and Liard, 1987; Gerstberger et al., 1987). However, the AVT concentration shown to stimulate water intake in ducks or to activate SFO neurons in vitro was of the order of 2700 pM VP. In contrast, continuous infusion of AngII which resulted in a plasma concentration of about 1400 pM, produced a much higher drinking response compared to AVT. Furthermore, electrophysiological data showed a more than 10-fold higher sensitivity of SFO neurons to AngII as compared to AVT. Therefore we hypothesized that the drinking observed in ducks after iv. administration of AVT was most likely due to stimulation of receptors normally accessed by a central vasotocinergic fiber input to SFO, since the AVT concentration in the synaptic cleft could certainly reach effective concentrations higher than the endogenous physiological plasma levels (Schmid et al., 1995a,b; Schmid and Simon, 1996).

In summary, in rats and ducks AVT/VP affects the activity of the majority of SFO neurons. While the exclusively excitatory effect of AVT on duck SFO neurons correlates well with the dipsogenic effect of AVT in this experimental model, the heterogeneous action of VP observed in the rat SFO will hamper the future experimental approach to its possible dipsogenic action in mammals. Although peripheral AVT alone does not initiate drinking in rats and ducks, the additive effect of AngII and AVT observed on individual SFO neurons (Schmid et al., 1995b) might contribute to a facilitation of water intake when plasma concentrations of both peptides are elevated simultaneously during dehydration or when the brain-intrinsic vasopressinergic innervation of SFO cells is co-activated. The responsiveness of other control systems assumed to be influenced via the SFO might depend on the interaction of local AVT/VP and AngII in a similar fashion. In line with this conclusion are experiments showing that combined application of sub-pressor doses of VP together with AngII caused a significant rise in blood-pressure (Ishikawa et al., 1984).

Area postrema

The AP, together with the NTS, makes up the viscerosensory components in the control of autonomic responses provided by the medulla oblon-

gata. While it is unlikely that VP affects AP neurons from the ventricular side, synaptically released and blood-borne VP can influence AP cells. To what extent blood-borne or synaptically released VP affects AP function is currently not known (Smith et al., 1994; Smith and Ferguson, 1996), but in contrast to the SFO, blood-borne VP may have a more pronounced influence on AP neurons, due to the higher sensitivity of AP neurons to VP (Cai and Bishop, 1995; Anthes et al., 1997).

Physiological effects of VP on the area postrema

Various physiological functions have been attributed to the AP including the regulation of food-intake, cerebrospinal fluid production, fluid homeostasis, metabolism and most notably emesis and cardiovascular regulation (Borison et al., 1984; Miselis et al., 1987a,b). Emesis one of the first described physiological functions of the AP shows strong species dependence. Rodents for example do not vomit in contrast to dogs, ferrets and humans (Borison et al., 1984). Carpenter et al. (Carpenter et al., 1984; Carpenter and Briggs, 1986) showed that peripherally applied VP, among 27 other substances tested, caused vomiting in dogs and excited silent AP neurons recorded from dogs in vivo (Carpenter et al., 1988). From this and other studies, it was concluded that substances which activate AP neurons and elevate cAMP may likely be involved in initiating the vomiting reflex. A simple (but now disproved) explanation for the inability of rats to vomit in response to apomorphine, AngII and glutamate proposed that these substances were not able to excite neurons in the AP of these species, as shown by Brooks et al. (1983). In contrast, dogs respond to such drugs with AP excitation and show sensitive vomiting reflexes (Brooks et al., 1983; Carpenter et al., 1988). However, several groups have since reported that VP, AngII, glutamate and many other substances can, in fact, excite AP neurons in rats and rabbits (Cai et al., 1994; Smith et al., 1994; Cai and Bishop, 1995; Sun and Ferguson, 1996). These data provide evidence that the excitation of AP neurons by an emetic neuromodulator alone, at least in rodents, is insufficient to evoke vomiting. It has been proposed that vagal afferent sensory signals from the gastrointestinal tract and circulating information sensed by the AP must be integrated within a brainstem motor area dubbed the 'vomiting center' in order to initiate the motor act of emesis (Borison et al., 1984; Borison, 1989). While emesis may be limited to those species having the motor capacity to vomit, AP-mediated nausea and food aversion (Miselis et al., 1987) is likely expressed by all species.

More recent physiological studies have focused on the role of the AP in cardiovascular function (Bishop and Hay, 1993; Smith et al., 1994; Hegarty et al., 1996). The effect of VP on cardiovascular functions is complex. VP activates V_1 receptors in the vasculature which mediate vasoconstriction and increase in total peripheral resistance (Cowley and Liard, 1987). Furthermore, VP activates V_2 receptors in the kidney to stimulate the insertion of water channels in VP-sensitive renal collecting duct cells resulting in increased water reabsorption. In addition to its peripheral actions, blood-borne VP acts centrally to sensitize baroreflex control of heart rate (HR) and sympathetic tone such that a given rise in arterial pressure results in a greater reflex inhibition of HR and sympathetic activity. This sensitization effect also appears to differ between species, as dogs and rabbits (Applegate et al., 1987; Cowley and Liard, 1987; Bishop and Hay, 1993) clearly show sensitized reflexes, while the effect is controversial in rats (Webb and Osborn, 1986; Osborn et al., 1987). In rats, VP acting on the AP was shown to cause a distinct bradycardia with little effect on sympathetic activity in one study (Peuler et al., 1990). Using lesioning techniques and direct microapplication of VP, the AP has since been identified as the central target responsible for the baroreflex sensitization effect of VP in various species (Brizzee, 1990; Ferguson, 1991, 1992). In an elegant study on rabbits, Undesser et al. (1985) showed that infusions of the vasoconstrictor agent, phenylephrine, caused pressure-dependent reflex decreases in renal sympathetic nerve activity (RSNA) and HR, while VP produced abrupt decreases in RSNA and HR in intact rabbits, prior to detectable increases in MAP. This indi-

cates that the vasoconstrictor effect of VP is buffered by a centrally mediated rapid and enhanced reflex inhibition of sympathetic activity, which prevents a rise in MAP in response to low doses of VP. In AP-lesioned animals, VP and phenylephrine caused the same pressor-dependent decrease in RSNA and HR. The effect of VP on the arterial baroreflex has been shown to be V_1 receptor mediated (Hasser and Bishop, 1990), as AP injection of a V_1 receptor antagonist blocked the ability of blood-borne VP to enhance the inhibition of the baroreflex (Bishop and Hay, 1993). In contrast, Lowes et al. (1993) have reported in rats that microapplication of VP into the AP was able to increase MAP via V_1 receptor activation, but V_2 receptor activation by dDAVP caused a reduction in MAP. Microinjection of VP into the AP in rabbits was shown to cause a dose-dependent decrease in RSNA and HR, without altering MAP, and the reflex bradycardic response to i.v. phenylephrine was also augmented after injection of VP into the AP (Hasser and Bishop, 1990). Bishop and coworkers recently proposed a model in which they suggested that VP acts on the AP to shift the operating point of the baroreflex to lower pressures resulting in a lower sympathetic outflow at a given arterial pressure (Bishop and Hay, 1993).

Blood-borne AngII also has been found to influence baroreflex control via the AP (Bishop and Hay, 1993; Fink et al., 1987; Otsuka et al., 1986; Lowes et al., 1993). In contrast to VP, however, AngII acts on the AP to decrease the sensitivity of the baroreflex response in various species. Specifically, the reflex bradycardia elicited by i.v. AngII injection was attenuated in comparison to responses evoked by phenylephrine-induced pressor responses. It has since been suggested that AngII inhibits the reflex cardiac parasympathetic activation that normally accompanies acute pressor responses (Scroop and Lowe, 1969; Matsukawa and Reid, 1990). In contrast to VP, AngII is thought to act via the AP to shift the baroreflex to higher pressures (Bishop and Hay, 1993). While the attenuation of baroreflex control of HR is observed after acute AngII exposure, changes in sympathetic baroreflex control requires chronic AngII exposure. The AP also appears to be critical for the development of chronic AngII-dependent hypertension (Otsuka et al., 1986; Fink et al., 1987), whereas a long-term elevation of plasma VP has not been implicated in the development of hypertension (Pawloski et al., 1989).

Cellular effects of VP on neurons in the area postrema

Several in vivo and in vitro studies have attempted to elucidate the cellular basis for the AP-mediated responses (Table 4). However, inconsistencies in the data have so far precluded any clear understanding of a general mechanism underlying the physiological responses known to be mediated by the AP. Based on in vivo data showing that AP-specific cardiovascular responses can be elicited by stimuli which are only excitatory on neurons, such as electrical stimulation or local injection of glutamate (Hasser et al., 1987; Ferguson and Marcus, 1988), it was predicted that substances which decrease (AngII) or increase (VP) the baroreflex inhibition should have corresponding excitatory and inhibitory effects on AP neurons (Ferguson and Bains, 1996). However, experiments using exclusively excitatory stimuli showed that the elicited responses were strongly dependent on the species, the intensity of the stimulus and the exact location of the stimulus electrode. In dogs, electrical stimulation of the AP was shown to increase HR, MAP and cardiac output (Barnes et al., 1979; Ferrario et al., 1987). In rats however, low frequency stimulation of the medial AP caused a marked decrease in HR and MAP which could be reversed by lowering the stimulating electrode further into the dorsal NTS (Hasser et al., 1987; Ferguson and Marcus, 1988). Presently, it is thought that an excitation of AP neurons by low frequency electrical stimulation or injection of glutamate causes an inhibitory effect on HR, RSNA and MAP (Ferguson and Marcus, 1988; Bishop and Hay, 1993; but see Hegarty et al., 1995).

A prevalence of 84% excitation among AP neurons responding to circulating AngII in vivo (Papas et al., 1990) and a prevalence of 54% inhibition among AP neurons responding to circulating VP in vivo (Smith et al., 1994) initially suggested such a mechanism. However, in vitro extracellular recordings from AP brain slices have demonstrated

predominantly excitatory effects of VP on AP neurons (Table 4), suggesting that this is a direct action (Cai and Bishop, 1995; Lowes et al., 1995). Recently, it was confirmed that the excitatory effect of AngII and VP on neurons in AP slices persisted after synaptic blockade with a low Ca^{2+} and high Mg^{2+} solution indicating a direct postsynaptic action (Lowes et al., 1995; Sun and Ferguson, 1996). On the other hand, the low, sometimes absent (Carpenter et al., 1988; Cai and Bishop, 1995) spontaneous activity of AP neurons in vivo suggests a major inhibitory synaptic input to AP neurons. But even in the isolated AP slice inhibitory synaptic interactions are still present, as demonstrated by experiments which showed that 18% of cells responded to the excitatory amino acid, glutamate, with a mono- or biphasic inhibitory response (Sun and Ferguson, 1996), an effect which can only be explained by local inhibitory synaptic interactions.

The similar direct excitatory effect of VP and AngII on the majority of AP neurons recorded in vitro failed to provide a cellular basis for the opposite effect both peptides have on baroreceptor reflex. Therefore the opposing effects must be mediated by elements downstream from the AP, possibly by differential stimulation or inhibition of fractions of neurons in the NTS, the major projection site of AP neurons. This, however, would imply that VP and AngII must act on different fractions of AP neurons, because it is unlikely that excitation of the same neurons by AngII and VP would cause inhibitory as well as excitatory responses in postsynaptic cells. Using the same comparative approach, which has been used successfully in the past to reveal the physiological relevance of various neuromodulators in the SFO (Schmid and Simon, 1992, 1996; Rauch et al., 1997). Cai and Bishop (1995) compared the effect of VP and AngII on identical AP neurons, but as reported for the rat SFO (Anthes et al., 1997) they did not find a conclusive correlation between VP and AngII responsiveness.

A recent in vitro study investigated the activity of NTS neurons after microinjecting VP and AngII into the AP and found that VP caused mainly excitatory and AngII mainly inhibitory responses (Cai et al., 1994). Using calcium imaging techniques Hay et al. (1993) showed that each of 6 neurons from the AP/NTS region tested responded with an increase in calcium after AngII as well as VP. While these data suggest that receptors for both peptides are expressed in the same cells, it does not allow conclusions as to whether the cells are excited or inhibited by the peptides. Recently Hegarty et al. (1996) investigated the effect of circulating AngII and VP on blood pressure -sensitive and -insensitive neurons in the anesthetized rat and found that AngII excited 17 and inhibited 15 of 74 neurons tested and VP excited 13 and inhibited 21. The inhibitory and excitatory effects of both peptides usually, but not always, correlated well with the baroreceptor sensitivity of the same cells and the type of VP and AngII responsiveness of the same cells did not show any significant correlation. Therefore, the authors stated that the 'mixed influence of these peptides does not clearly correlate with the reported attenuation and enhancement of the baroreflex by circulating AngII and VP respectively' (Hegarty et al., 1996). As shown in Table 4 recordings in vivo and in vitro revealed significant differences in the frequency of inhibitory and excitatory responses of VP on NTS neurons.

In summary, the cellular responses of AP and NTS neurons to VP do not provide a clear picture as to how VP's physiological effects are transduced in the AP. The heterogeneous effects of VP on AP and NTS neurons illustrates the difficulty in identifying a clear role for this peptide, particularly as it relates to physiological responses as complex as emesis or the baroreceptor reflex. Further studies stimulating specific inputs to neurons in the AP and NTS in vivo might help to identify the cellular basis for VP-mediated effects on the AP.

Organum vasculosum of the lamina terminalis

Effects of VP on cells from the organum vasculosum of the lamina terminalis

To our knowledge, no electrophysiological data are available which address the effect of VP on OVLT neurons. The currently available data describing the effect of VP and its degradation product, VP 4–9, on calcium transients have already been described (Table 1). Although the increase in intracellular calcium concentrations

Table 4
Responsiveness of AP and NTS neurons to VP in electrophysiological studies performed in different species

Neurons responsive to VP			Tested	Species	Reference
Excitation (%)	Inhibition (%)	No response (%)			
AP in vivo studies					
38	46	16	107	Rat	Smith et al., 1994
50	–	50	10	Dog	Carpenter et al., 1988
AP in vitro studies					
53	15	32	47	Rabbit	Cai and Bishop, 1995
64	6	30	79	Rat	Lowes et al., 1995
NTS in vivo studies					
17	27	56	77	Rat	Hegarty et al., 1996
NTS in vitro studies					
49	19	32	57	Rat	Cai et al., 1994

observed in response to VP and VP 4–9 suggests an excitatory effect on OVLT neurons, a direct inhibitory effects of these peptides, which has been observed in the SFO (Schmid et al., 1995a,b; Anthes et al., 1997), might also be mediated by an increase in intracellular calcium, e.g., by activation of Ca^{2+}- dependent K^{+}-currents. The small size of the OVLT and the fact that it is in direct cellular contact with the adjacent hypothalamic areas necessarily results in 'contamination' of primary cultures with cells located outside the OVLT. It is likely that the failure of direct microapplications of VP into the OVLT of guinea pigs to provide consistent physiological responses, is due to the same problems (Roth and Zeisberger, pers. commun.).

Physiological effects of VP in the organum vasculosum of the lamina terminalis

The OVLT has been implicated in the regulation of body-fluid homeostasis and in fever (Blatteis, 1992; Zeisberger and Merker, 1992). Although immunohistochemical evidence has demonstrated the presence of vasopressinergic fibers and cells in the OVLT, the functional role of VP in the OVLT has not been studied so far, primarily due to the experimental difficulties described above (Palkovits, 1987; Zeisberger and Merker, 1992).

Recent electrophysiological recording provided convincing evidence for the osmosensitivity of OVLT neurons in rats and ducks (Bourque et al., 1994; Müller et al., 1994). In rat hypothalamic slice preparations (Richard and Bourque, 1995) it was shown that osmotically stimulated OVLT neurons activate VP-synthesizing neurons of the SON via glutamatergic input. It has repeatedly been shown that VP is released into the circulation during osmotic stimulation as well as within the brain to cause antidiuretc, natriuretic and other centrally-mediated effects (Buijs, 1978; Landgraf and Ludwig, 1991; Bourque et al., 1994). Vasopressinergic neurons in the SON have also been shown to be osmosensitive themselves which offers another possibility for osmotically induced release of VP in the hypothalamus and possibly OVLT (Oliet and Bourque, 1994; Bourque et al., 1994). The well documented vasopressinergic input from the SCN suggests a circadian influence on OVLT-mediated responses. It is possible that osmotically-mediated diurnal water-intake is regulated by such input, but direct evidence for such an effect is still lacking (Buijs, 1978).

In addition to its osmosensory function, the OVLT is thought to play a role in the development

of fever. Due to its open BBB, the OVLT may be a central target for cytokines which are produced by monocytes and macrophages in response to exogenous pyrogens (Blatteis, 1992). Cytokines such as IL-1β bind to luminal receptors on endothelial cells of the heavily vascularized OVLT and stimulate the production of prostaglandin E2 (Cao et al., 1996), which represents the pyrogenic mediator for hypothalamic neurons involved in thermoregulation, although other mechanisms for the transmission of the cytokine signal to the brain may exist as well (Sehic and Blatteis, 1996; Goldbach et al., 1997). It has been found that fever is suppressed by the action of VP, an endogenous antipyretic substance, liberated within the brain during fever (Cooper, 1987; Zeisberger, 1990). This antipyretic action is caused by vasopressinergic input to the ventral septal area, which is known to project to hypothalamic neurons relevant in thermoregulation and into the OVLT. It has been suggested by Zeisberger and Merker (1992) that these septal efferents may contact vasopressinergic neurons within the OVLT and thus add to the central antipyretic effect, possibly by altering the vascular permeability and thus the access of interleukins to their receptors.

Conclusions

One of the best investigated functions of the sensory CVOs is the dipsogenic effect of AngII in the SFO. Here data from receptor autoradiography, immunohistochemistry, electrophysiology, lesion studies and other physiological experiments clearly support the notion that the stimulation of AngII receptors in the SFO leads to water intake. The role of VP in the sensory CVOs, however, seems to be more complex. The autoradiographic localization of receptors and the immunohistochemical detection of VP is less consistent and electrophysiological data in mammals, when available, show excitatory as well as inhibitory responses to VP. In less complex systems, like the duck, the solely excitatory responses in SFO neurons and the observed drinking response to AVT match to a closer correlation. The cell culture findings show that different VP receptor subtypes and receptors for VP fragments are expressed on neurons as well as on astrocytes derived from sensory CVOs. These data might be used to develop new experimental approaches, a straightforward model for the functional role of VP in the fascinating 'windows to the brain' as yet appears difficult to find.

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CHAPTER 3.1.6

Steroid hormone regulation of vasopressinergic neurotransmission in the central nervous system

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Vasopressinergic neurotransmission is intimately linked to steroid hormone signaling. Both arginine[8] vasopressin (VP) and the extrahypothalamic VP V_{1a} receptors are regulated by steroid hormones. Here, we present work that has been done in our laboratory, investigating mechanisms underlying steroid hormone effects on the expression of both VP and its primary receptor in the brain, the VP V_{1a} receptor. Data on VP receptors, their coupling to second messenger pathways, their localization in brain, and their regulation by peptide exposure are discussed. We also cover the regulation of the V_{1a} receptor by adrenal hormones, and the molecular basis of this effect. Evidence for the existence of other receptors for VP in the brain is presented. Lastly, the regulation of the VP peptide by gonadal hormones is discussed at the transcriptional level in the rodent brain. Finally, the potential significance of the 'cross-talk' between the vasopressinergic system and the steroid hormone system is addressed.

Vasopressin receptors

Introduction

Two distinct types of VP receptors have been identified and termed V_1 and V_2. These receptors are differentiated both by ligand specificities and by receptor coupling mechanisms. The V_2 type receptors are expressed primarily in the renal collecting ducts, and promote water permeability (Handler and Orloff, 1981; Elalouf et al., 1986) and stimulation of sodium, calcium, magnesium and chloride reabsorption in the ascending limb (Wittner et al., 1988). These receptors are coupled to Gs-type proteins and stimulate adenylyl cyclase (Bockaert et al., 1973). V_1 receptors are coupled to various pertussis toxin-sensitive G proteins including Gq, and Gi or Go (Swank and Dorsa, 1990) and activation of several second messenger pathways such as phospholipase A_2, C, as well as phospholipase D through the activation of ligand gated calcium channels (Briley et al., 1994). V_1- type receptors are further subdivided into the V_{1a} and V_{1b} subtypes, based on ligand specificities and tissue localization. The V_{1b} receptors were originally identified in the anterior pituitary (Antoni, 1984; Jard et al., 1986; Arsenijevic et al., 1994) and are involved in ACTH secretion, while V_{1a} receptors are expressed in the liver, where they promote glycogenolysis; smooth muscle vasculature, where they promote vasoconstriction; and also in the brain (Keppens and DeWulf, 1975; Pittman et al., 1982; Antoni, 1984; Jard et al., 1986; Guillon, 1989). Brain VP receptors have been characterized as pharmacologically similar to the peripheral V_{1a} type based on agonist and antagonist specificities (Dorsa et al., 1988; Phillips et al., 1988). Agonist binding to brain VP receptors induces phosphoinositide hydrolysis which further supports their similarity to the V_{1a} type VP receptor (Shewey and Dorsa, 1988). Therefore, identification of factors important in regulation of expression of this recep-

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tor could have implications for understanding the role of VP in metabolic and cardiovascular function as well as CNS neurotransmission.

VP binding site localization and pharmacology

Vasopressin's role as a neurotransmitter or neuromodulator in the CNS implies the presence of neuronal binding sites either on VP neurons themselves or on other neurotransmitter systems. [^{3}H]VP was the first radiolabelled VP analog available for use to determine the localization of the VP receptor protein in rat brain and peripheral tissues, and to compare their pharmacologic characteristics. Binding of [^{3}H]VP was noted in the nucleus tractus solitarius (NTS) of the brain stem in addition to a classical peripheral target tissue of VP action, the kidney (Dorsa et al., 1983). This represented the first autoradiographic evidence for the presence of VP specific binding sites in the brain which might mediate the previously reported effects of VP on blood pressure. Upon further pharmacologic characterization of the hindbrain site, it was found that selective antidiuretic compounds had higher affinity for the renal medullary site than they did for the hindbrain site, which seemed to have binding characteristics more similar to the pressor sites, indicating that although both the kidney and brain sites bound [^{3}H]VP with what appeared to be similar kinetics, they were in fact different receptor subtypes (Cornett and Dorsa, 1985). These data in conjunction with those of other laboratories, lead to the hypothesis that of the two different receptor subtypes for VP, those in the brain most closely resembled the Ca^{2+} dependent V_1 receptor which mediates the peripheral pressor and glycogenolytic responses of VP in the smooth muscle and liver respectively, and not the cAMP-dependent V_2 receptor which mediates the antidiuretic response of VP in the renal medulla. The availability of a wide variety of synthetic VP analogs provided support for this hypothesis, which distinguish between the two receptor subtypes (Michell et al., 1979) both by binding and biological criteria. Somewhat later, other regions in the brain were also found to contain binding sites for VP including: the lateral septum of rat brain, nucleus accumbens, diagonal band of Broca, the nucleus intersitialis stria terminalis (Baskin et al., 1983), and the central and medial aspects of the amygdala (Dorsa et al., 1984) using the tritiated VP compound. Taken together, these data allowed the construction of a map of VP receptor distribution in the brain, and began to clarify the pharmacology of the receptors; namely, the sites in the liver and the CNS (V_1) were distinct from the V_2 type renal receptors.

Ligand specificity and signal transduction pathway coupling of the rat brain VP receptor was further investigated in this and other laboratories. Scatchard analyses revealed that in septal tissue, VP binding was best described by a single class of binding sites which exhibited a K_d for VP binding of 1.7 nM (Shewey and Dorsa, 1988). Displacement studies revealed that the septal site was most similar to the V_1 type (pressor) receptor which mediated the hydrolysis of inositol phospholipids in response to VP exposure. This effect was blocked by the V_1 antagonist, d$(CH_2)_5$ Tyr$(Me)_2$ VP. This suggested that the septal V_1 type VP receptor was coupled to inositol phospholipid metabolism potentially through phospholipase C (PLC) activation via a G-protein linked receptor event. This was investigated using non-hydrolyzable GTP analogs which significantly increased the K_d of VP for its receptor, and pertussis toxin which reduced the specific binding of VP by over 50% (Swank and Dorsa, 1990). These data indicated that in fact, the septal V_1 receptor was coupled to PLC via a Gi- or Go like GTP binding protein, and was in this respect, unlike the hepatic V_1 receptor that was shown to be coupled to PLC by a pertussis toxin insensitive G-protein (Blackmore et al., 1988).

Regulation of VP binding sites

Having mapped the sites in the brain where VP binding was most obvious, and the general pharmacology of these receptor sites, it was of interest to determine whether these binding sites exhibited autoregulatory responses comparable to other neurotransmitter receptors. That is, are they modulated by the presence of VP itself. VP research has benefited by the existence of a naturally occurring 'knockout' model, the Brattleboro (BB) rat. The genetic defect is due to a single nucleotide deletion

within the gene encoding pre-propressophysin (Schmale and Richter, 1984) rendering it unable to synthesize VP or its related neurophysin in the brain. This mutation leads to inefficient translation of the mRNA for VP, and the animals have as a result, undetectable levels of VP in their plasma, hypothalamus or pituitary (Dorsa and Bottemiller, 1982). Heterozygous animals have about 50–60% of the normal concentration of VP, but maintain relatively normal fluid and electrolyte homeostasis (Mohring and Mohring, 1975). Although VP is not present in these animals in detectable levels of any consequence, binding sites for [^{3}H]VP in the BB rat are present in the brain, and are similar in distribution to those of the parent Long–Evans strain (Petracca et al., 1986), indicating that presence of VP binding sites is independent of the presence of VP itself. The VP receptors of the BB rat are however, present in higher numbers in the septum and liver. In the septum, they also exhibit a lower affinity for the VP peptide (Shewey and Dorsa, 1986). The affinity of the renal or hepatic receptors in the BB rat are not different from heterozygotes, nor do the number of renal receptors change. This indicates that the presence of the ligand might regulate the number of binding sites for VP in the liver and in the brain, and also might in the brain, alter the affinity of the receptor for the ligand.

In another study, our laboratory investigated the means by which the receptors for VP are regulated by one of the stimuli which is known to control the peripheral release of VP itself, osmotic stimulation. The central release of VP has been shown to be increased by extreme osmotic stimulation, and thus the effect of osmotic stimulation on the binding characteristics of [^{3}H]VP in the brain and in the periphery were evaluated. Surprisingly, no significant change in the binding characteristics of VP in the septum, dorsal hippocampus or in the amygdala was noted, but there was a significant reduction in the number of binding sites in the kidney, with no effect on affinity (Landgraf et al., 1991). These results suggest that the receptors for VP in the CNS are resistant to the regulatory effects of acute VP exposure. Since acute VP exposure had no effect on VP binding sites in the CNS, a follow up study evaluated the effect of chronic VP and VP-antagonist exposure on VP binding sites in the brain. Neither VP nor the V_1 receptor antagonist d(CH_2)$_5$-Tyr-(Me)-VP altered total binding site concentration in the septum or in the amygdala, but the antagonist did reduce the affinity for both the agonist and antagonist in both regions (Swank and Dorsa, 1991). Therefore, it can be concluded that acute exposure to ligand does not appear to promote up- or down-regulation of vasopressinergic receptors, unlike that seen with other serpentine receptor systems. Chronic ligand deprivation as is seen in the Brattleboro rat however, may influence binding site density and affinity.

Studies were then performed to investigate the downstream consequences of V_1 receptor up-regulation in the Brattleboro rat. Vasopressin-induced phosphatidylinositol (PI) hydrolysis was measured in the septum of homozygous and heterozygous BB rats, in order to determine if the increase in binding site number observed in homozygous BB rats results in an increased post-receptor response of the tissue (Shewey et al., 1989). The homozygous rats showed a larger increase in PI hydrolysis in response to VP in the septum than did the heterozygous animals, which had a lower amount than the parental Long–Evans animals. These results suggested that the enhanced number of binding sites present in the homozygous animals compared to the heterozygous animals also led to differences in post receptor response, but not one which was greater than that seen in the parental strain. Further investigation revealed that chronic exposure to VP resulted in a decrease in septal receptor number which was accompanied by a decrease in post-receptor PI hydrolysis in the homozygous BB rat, but not in the heterozygous animal (Shewey et al., 1989). This apparent VP-induced receptor down-regulation also results in receptor desensitization in the septum of homozygous animals. The modulation of VP receptor number and post-receptor signalling events by VP itself do not occur in either the parental strain or in the heterozygous animals, which themselves appear to be most like the wild-type animal. Therefore, the regulation of receptor events noted in the homozygous BB animals probably do not reflect the same regulatory events expected to occur in the wild-type animal.

VP binding sites during development

Ontogenetic expression of binding sites for VP is highly dynamic. The regional distribution for [^{3}H]VP binding changes markedly throughout early postnatal development. Binding sites in the septum increase slowly to attain adult levels in the dorsal and lateral septum, while binding in the caudate nucleus, hippocampus and cingulate gyrus display intense levels of labelling during early development which disappear by adulthood (Petracca et al., 1986). [^{3}H]VP binding did not change however, either during early development nor in adulthood, in the central nucleus of the amygdala. In an attempt to further characterize the binding site present in the cingulate gyrus-dorsal hippocampus of the developing rat brain, our laboratory undertook the pharmacological characterization of this receptor (Szot et al., 1989). In comparing the postnatal day 8 pup cingulate gyrus with that of the adult, an increase in the total number of binding sites was observed in the adult, but the affinity of the adult sites were greatly reduced in comparison to those present in the developing brain. The pharmacologic characterization of both the adult and pup cingulate gyrus sites were similar, with respect to agonist/antagonist profiles, but differed markedly from the pharmacologic profile of the receptor present in the adult septum. The pup cingulate gyrus receptor had a reduced affinity for V_1 receptor antagonists, indicating that it is a pharmacologically distinct site from the V_1 receptor present in the septum. A subsequent study evaluated the effect of VP administration or deficiency on the septal V_1 receptor during the development of the rat brain. VP deficiency was investigated using homozygous BB rats. Again, as would be expected from the data presented thus far, there was no change in septal or kidney receptor after 7 days of VP administration beginning at postnatal day 1 (Szot et al., 1992). There was however, a significant increase in binding site density, but not affinity, in the liver which was maintained into adulthood. In the homozygous BB rat, the hepatic receptors showed an increase in density while the heterozygous hepatic receptors were reduced in numbers compared to the parental Long–Evans rat. Thus, regardless of when the agonist is administered, either early postnatally or in the adult state, or either chronically or acutely, VP does not appear to regulate number or affinity of VP receptors in the septum of rat brain, while it may do so in hepatic tissue.

In order to further characterize VP binding sites present in the cingulate gyrus of the postnatal day 8 pup brain, we undertook in situ hybridization experiments using oligonucleotide probes to the then recently cloned VP receptors, V_{1a}, V_{1b} and V_2. We reasoned that if the receptor present in the cingulate gyrus was one of the known VP receptors, then a mRNA distribution pattern similar to the pattern observed with [^{3}H]VP binding might be apparent. While mRNA distribution was not observed to be identical to that of the binding at postnatal day 8, hybridization positive cells for two of the three VP receptors were present in the cingulate gyrus. The developing rat brain appears to express mRNA for both the V_{1b} (Fig. 2) and V_2 (Fig. 3) receptors. V_2 receptors are not thought to be expressed in the adult rat brain. Additionally, mRNA for the V_{1a}R was also detected in regions where binding for ^{3}H VP has been documented during development of the rat brain (Fig. 1), namely the lateral septum, bed nucleus of the stria terminalis and hippocampus. More recent data also suggests that OT receptor mRNA and binding sites may also be present in the cingulate gyrus and caudate nucleus during early development (Bale et al., unpublished observations).

mRNA for VP V_{1a} receptor in rat brain

After the molecular cloning of the rat V_{1a} receptor (V_{1a}R) gene (Morel et al., 1993), we and others mapped the localization of the mRNA for the V_{1a}R in the adult rat brain (Ostrowski et al., 1994; Szot et al., 1994). The distribution of the mRNA throughout the brain is in part consistent with those regions previously shown to contain binding sites for radiolabelled vasopressin. These regions include the olfactory nucleus, Islands of Calleja, hippocampus, suprachiasmatic nucleus, arcuate nucleus, and the ventromedial nucleus (Szot et al., 1994). Certain regions of the rat brain were not as rich in V_{1a} mRNA content as would be expected using a probe complementary the 5′ untranslated region

of the $V_{1a}R$ gene, especially the septum and the amygdala which had been shown by us and others to contain a high density of VP binding sites (Baskin et al., 1983; Biegon et al., 1984; Brinton et al., 1984; Dorsa et al., 1983, 1984). Interestingly, Ostrowski et al. (1994), using a riboprobe spanning the putative fifth transmembrane domain through the seventh transmembrane domains of the predicted protein structure, detected mRNA for the $V_{1a}R$ in both the central nucleus of the amygdala and the lateral septum, indicating that there may be differential mRNA isoform expression of the $V_{1a}R$ gene in the brain. To this end, we have performed Northern blot analyses of mRNA from various brain regions and the liver, using probes spanning different regions of the predicted protein structure of the rat V_{1a} receptor. We have detected two major bands, one at the previously reported size of 2.1 kb (Morel et al., 1993) and the other at approximately 6.2 kb (Fig. 4). These bands are detected with different intensities depending upon the probes

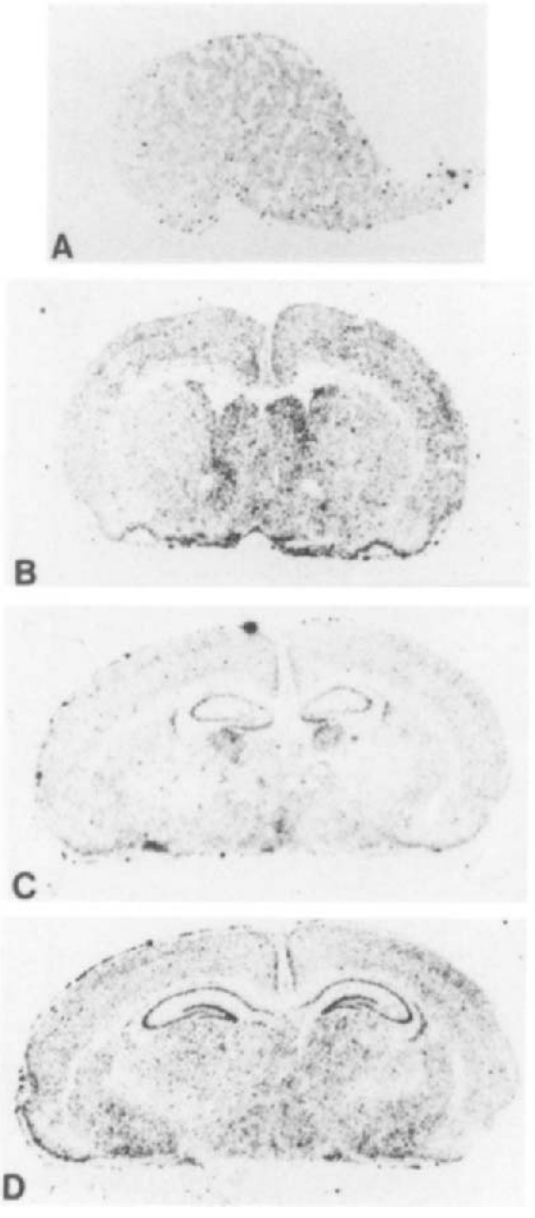

Fig. 1. Film autoradiograms indicating localization of $V_{1a}R$ mRNA by in situ hybridization in coronal sections from the postnatal day 8 developing rat brain. (A) Liver; (B–D) coronal sections through postnatal day 8 rat brain.

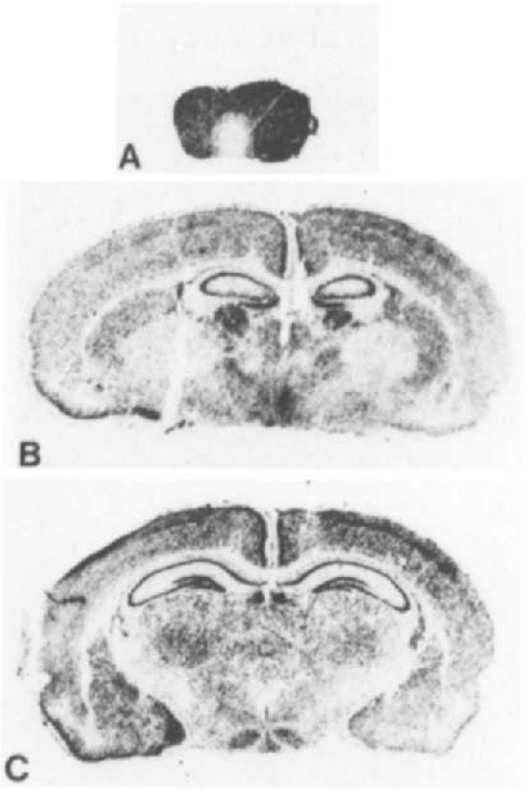

Fig. 2. Film autoradiograms indicating localization of $V_{1b}R$ mRNA by in situ hybridization in coronal sections from the postnatal day 8 developing rat brain. (A) Pituitary; (B,C) coronal sections through postnatal day 8 rat brain.

used. Oligonucleotides directed against the 5′ untranslated region, and 1st and 2nd extracellular loops of the predicted protein structure (N-terminus) indicate a band on a Northern blot of approximately 2.1 kb, the same size as that reported for the hepatic $V_{1a}R$ cDNA cloned by Morel and colleagues. However, when using a riboprobe spanning transmembrane domains 5 through 7 (C-terminus), while the 2.1 kb band is detected, the major band appears to be much larger, approximately 6.2 kb. Interestingly, Ostrowski et al. (1992) reported that

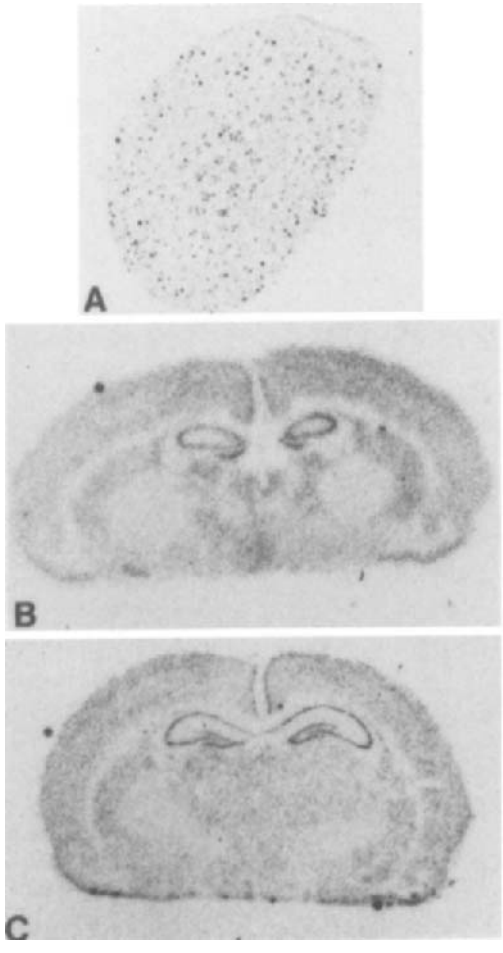

Fig. 3. Film autoradiograms indicating localization of V_2R mRNA by in situ hybridization in coronal sections from the postnatal day 8 developing rat brain. (A) Kidney; (B,C) coronal sections through postnatal day 8 rat brain.

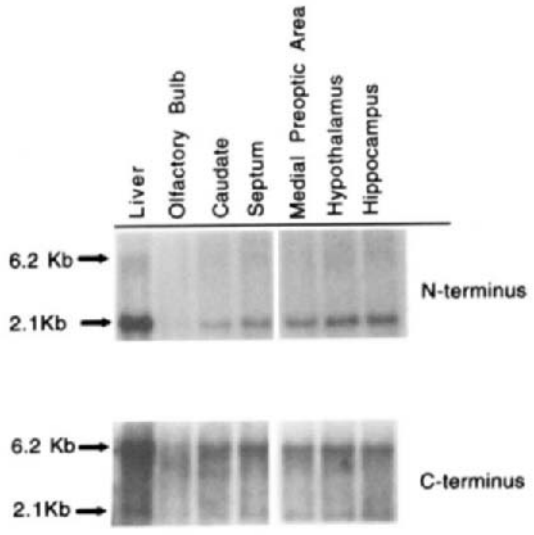

Fig. 4. Northern blots indicating size of $V_{1a}R$ mRNA detected in various brain regions using a probe from the N-terminus (upper panel) or C-terminus (lower panel) of the $V_{1a}R$ cDNA.

one major band at 2.2 kb was detected in the liver and kidney, and upon overexposure, a second very minor band was detected at 2.8 kb when using a probe similar to that used in our studies. Unpublished observations from our laboratory indicate that there is also a difference in the localization of $V_{1a}R$ mRNA in the rat brain, depending upon the region of the $V_{1a}R$ cDNA used to generate probes for in situ hybridization (Szot and Dorsa, unpublished observations). Taken together, along with the data presented for the pup cingulate gyrus binding site, there is strong evidence to suggest that there are other isoforms of VP receptors present in the brain.

There are physiologically produced and behaviorally active fragments of VP, including VP 1–8, 4–9 and 4–8 that have been shown to be present in the hypothalamus, hippocampus, amygdala, and septum of rat brain, but not in the pituitary gland (Wang et al., 1986). Presumably these receptors mediate the effects of fragments of the VP peptide which are behaviorally active. Their effects are blocked by a $V_{1a}R$ antagonist, but these peptides have no effect on peripheral pressor responses. This suggests that the structural

requirements for activation of certain central VP receptors is different from that of peripheral VP receptors, although they both can be blocked by the same antagonist, furthering the concept that brain receptors do differ from peripheral receptors in some structural way. These metabolites appear to have highly potent effects on memory and related processes (Burbach et al., 1983). Unpublished observations from our laboratory indicate that VP 4–8 and 4–9 are unable to compete for binding with [^{3}H]VP or [^{125}I]V_{1a}R antagonist to brain V_{1a} receptors, further substantiating the existence of other VP receptors in the rat brain which might be directly responsible for some of the behavioral responses to VP.

Data from the laboratory of Barberis, indicate that in the hippocampus, there are at least two different sites for VP binding, one that has high affinity for vasopressin, OT and vasotocin, and another which exhibits high affinity for VP and vasotocin, and low affinity for OT (Audigier and Barberis, 1985; Tribollet et al., 1988). Since several groups have reported VP binding sites in the hippocampus, we undertook a study to determine whether a mRNA homologous to the hepatic V_{1a}R cDNA is present in the hippocampus of adult rat brain. After isolating hippocampal mRNA which was reverse transcribed, we used the polymerase chain reaction to amplify the V_{1a}R from this hippocampal cDNA, using oligonucleotides directed towards the 5th transmembrane domain and the 3′ untranslated region of the predicted mRNA structure. We amplified a gene product approximately 635 bp in length, corresponding to the previously cloned hepatic V_{1a}R. Upon sequencing of the 5′ and 3′ ends of this PCR product, we found that it was identical in nucleotide sequence to that of the hepatic receptor (Fig. 5). This suggests that although the pharmacology of the binding sites in the hippocampus is not identical to of V_{1a} receptors, at least some component of the observed binding can be attributed to expression of the V_{1a}R gene. It is possible that different tissues express different splice variants of the V_{1a}R gene, or that there are other genes encoding receptors which can bind VP analogs in addition to the V_{1a}R gene, e.g., OT receptors, V_{1b} receptors etc.

Characterization of the V_{1a}R gene promoter

In order to further understand how expression of VP receptors in the rat brain are regulated, our laboratory isolated a genomic clone encoding a rat VP V_{1a}R, sequenced approximately 2.2kb upstream of the putative transcription start site (Watters et al., 1996a). We hypothesized that transcriptional regulation of the receptor may be important, since evidence from the experiments performed thus far indicated that autoregulation at the level of the protein was not a prominent feature of CNS receptors. In the putative promoter region of our clone, we found numerous potential sites for transcription factor binding, including several glucocorticoid response elements. Treatment of WRK-1 cells which express a VP V_1 receptor, resulted in increased [^{3}H]VP binding resulting from an increase in the number of binding sites and mRNA for the V_{1a} receptor, which could be blocked by the addition of the glucocorticoid receptor antagonist RU 38486 (Watters et al., 1996b). The effects of glucocorticoids were also evaluated in vivo, in the rat brain. Adrenalectomy significantly reduced V_{1a}R density in the septum and bed nucleus of the stria terminalis (BNST) of the rat brain, and administration of dexamethasone or aldosterone were capable of restoring V_{1a}R density levels comparable to those in sham controls in the BNST (Watters et al., 1996b). Only dexamethasone was able to restore V_{1a}R binding in the septum of adrenalectomized animals. Additionally, two of the putative glucocorticoid response elements present in the 5′ flanking region of the rat V_{1a}R gene were shown to have the capacity to bind proteins present in nuclear extract containing glucocorticoid receptors, suggesting that these elements may provide the molecular basis through which brain VP receptors might be regulated by adrenal hormones. The resistance of VP receptor density to change in response to stimuli which are known to regulate the release of VP itself, and their stability in the presence or absence of VP or antagonists, suggest that adrenal hormones may play an important role in regulating the post-synaptic portion of the vasopressinergic signalling pathway. Thus, one possible mechanism by which the VP V_{1a} receptor is controlled, is at the transcriptional level by factors

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Brain V1aR    1 tctgctaccacatctggcgcaacatccgcggaaagacagcgtcctcgcga 50
                ||||||||||||||||||||||||||||||||||||||||||||||||||
Liver V1aR  971 TCTGCTACCACATCTGGCGCAACATCCGCGGAAAGACAGCGTCCTCGCGA 1020

Brain V1aR   51 cacagcaagggtgacaagggctctggggaggccgtgggtccctttcataa 100
                ||||||||||||||||||||||||||||||||||||||||||||||||||
Liver V1aR 1021 CACAGCAAGGGTGACAAGGGCTCTGGGGAGGCCGTGGGTCCCTTTCATAA 1070

Brain V1aR  101 ggggcttttggttacaccttgtgtcagcagcgtgaagagcatttcccgcg 150
                ||||||||||||||||||||||||||||||||||||||||||||||||||
Liver V1aR 1071 GGGGCTTTTGGTTACACCTTGTGTCAGCAGCGTGAAGAGCATTTCCCGCG 1120

Brain V1aR  151 ccaagatccgcactgtgaagatgacctttgtgattgtaa            189
                |||||||||||||||||||||||||||||||||||
Liver V1aR 1121 CCAAGATCCGCACTGTGAAGATGACCTTTGTGATTGTAAGCGCCTACATC 1170

Brain V1aR  190           aaattcgccaaggatgactccgatagcatgagccgaaaga 229
                          |||||||||||||||||||||||||||||||||||||||||
Liver V1aR 1371 CATGGCGCAGAAATTCGCCAAGGATGACTCCGATAGCATGAGCCGAAAGA 1420

Brain V1aR  230 cagacttcttattctaacaaccggagcccaacgaacagcactgggatgtg 279
                |||||||||||||||||||||||||||||||||||||||||||||||||||
Liver V1aR 1421 CAGACTTCTTATTCTAACAACCGGAGCCCAACGAACAGCACTGGGATGTG 1470

Brain V1aR  280 gaaggactcgcccaaatcttccaaatccatcagattcattcctgtctcca 329
                |||||||||||||||||||||||||||||||||||||||||||||||||||
Liver V1aR 1471 GAAGGACTCGCCCAAATCTTCCAAATCCATCAGATTCATTCCTGTCTCCA 1520

Brain V1aR  330 cttgagccccacgttcacgcagcccgactcttgggagggacttttgtgtc 379
                |||||||||||||||||||||||||||||||||||||||||||||||||||
Liver V1aR 1521 CTTGAGCCCCACGTTCACGCAGCCCGACTCTTGGGAGGGACTTTTGTGTC 1570

Brain V1aR  380 cagctagagatcgtaagagcagatgtgtttatgtgg 415
                |||||||||||||||||||||||||||||||||||
Liver V1aR 1571 CAGCTAGAGATCGTAAGAGCAGATGTGTTTATGTGG 1606
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Fig. 5. Sequence comparison of 5′ and 3′ ends of PCR product amplified from adult rat hippocampal cDNA to rat hepatic $V_{1a}R$ cDNA.

such as adrenal steroid hormones. This finding also suggests that sensitivity of post-synaptic elements of the VP system may be enhanced in stressful situations. Given the purported involvement of VP in learning and memory detailed in other chapters of this text, this may lead to enhanced consolidation or retention of information during stressful life events.

Hormonal regulation of the VP gene

Introduction

VP is involved in many diverse biological actions including water retention, glucose metabolism, blood pressure regulation, secretion of adrenocorticotropin hormone as well as events including enhancing performance in behavioral and learning and memory tasks. The vasopressinergic neuronal cell bodies are located in the supraoptic, suprachiasmatic and paraventricular nuclei of the hypothalamus in addition to the bed nucleus of the stria terminalis, the medial amygdaloid nucleus and perhaps the locus coeruleus. Their axons project to numerous regions of the brain including the olfactory tubercle, other nuclei within the hypothalamus, the septum, thalamic nuclei, and the hippocampus (DeVries et al., 1985). Based on the results of numerous behavioral experiments in rodents, and the diversity of the regions to which VP axons project, VP has been implicated in the consolidation and retrieval of learned information (De Wied, 1971, 1987).

Gonadal hormone dependency of VP gene expression

DeVries and co-workers elegantly made use of immunocytochemistry to document the existence of VP producing cells of the BNST of rat brain. Our laboratory utilized in situ hybridization to detect VP mRNA in the BNST, and have used it as a tool with which to evaluate the regulation of the VP peptide mRNA in this nucleus (Miller et al., 1988). To determine other extrahypothalamic neurons also synthesizing the neuropeptide, our laboratory again undertook in situ hybridization experiments to localize mRNA for VP in these cells. VP mRNA was detected in cells of the medial amygdala but not in the locus coeruleus, indicating that cells in the locus coeruleus may not synthesize the neuropeptide from the propressophysin gene itself (Urban et al., 1990), although by immunocytochemical methods, VP peptide was present in these cells.

It had been shown previously using immunocytochemical techniques (De Vries et al., 1981; De Vries and Al-Shamma, 1990) that VP neurons in the BNST of the rat brain were sexually dimorphic and sensitive to gonadal steroids. Our laboratory used in situ hybridization to evaluate the mRNA content for VP in this nucleus, and studies were undertaken to assess the ability of testosterone treatment to regulate appearance of mRNA for VP (Miller et al., 1989). Castration dramatically reduced the expression of VP mRNA in the BNST both by decreasing the number of cells expressing VP mRNA and by reducing the number of transcripts expressed per cell, which could be reversed by testosterone replacement. Additionally, a sex difference between male and female VP mRNA expression in this nucleus was noted (Miller et al., 1989). Males had a significantly greater level of expression per cell than did females, and males had more cells expressing VP mRNA than females indicating the sexual dimorphism observed by immunocytochemistry in the BNST and septum resulted from a higher biosynthetic capacity by males in these neurons. To investigate the ontogeny of this sex difference further, VP mRNA was studied in the BNST and amygdala of the postnatal developing rat brain (Szot and Dorsa, 1993). Males showed VP mRNA in the BNST at day 3 after birth and in the medial amygdala on day 5. Hybridization signal could not be detected in female brains in these regions prior to postnatal day 14 in the BNST and day 35 in the amygdala. This indicates that the development of the VP system is delayed in females compared to males, and that the sexual dimorphism of the VP system in adult rat brain is evident early in the developmental process.

Thus, while it was clear that testosterone modulated the expression of the VP gene in these neurons in the BNST, it was not known whether testosterone (T) directly, or the metabolites of T, estrogen and/or dihydrotestosterone (DHT) were responsible for this action. Thus, studies were undertaken to deter-

mine the mechanism by which testosterone accomplishes this modulation (Brot et al., 1993). T by itself in local implants, in the BNST elevated VP mRNA. DHT or estrogen alone only achieved partial restoration of VP mRNA content in the BNST, but when given together produced a synergistic effect on the mRNA comparable to T alone in a castrated animal. It remained to be determined whether these steroid hormones were acting to cause an increase in transcription of the VP gene, or perhaps acted by stabilizing the mRNA for VP. To this end, in situ hybridization was performed using both a probe detecting the mature VP mRNA transcript, and another directed at the unspliced primary transcript complementary to an intronic sequence of the VP gene in both the BNST and the medial amygdala (Szot and Dorsa, 1994). Six hours after castration, nuclear or primary transcript for VP was undetectable in both brain regions. Three hours of T replacement resulted in an elevation of primary transcript in both regions, whereas the cytoplasmic or mature transcript was slower to respond. A delay of approximately 3 days was noted in the BNST and amygdala for a return to a normal level of cellular expression, while it took 7 days for the number of transcripts expressed per cell to return to normal in both brain areas. These data suggested that the regulation of VP synthesis by testosterone at least in the BNST and the amygdala, resulted in part from steroid hormone modulation of transcription, since the increase in nuclear primary transcript preceded the increase in mature cytoplasmic transcript.

VP synthesizing cells of the BNST gradually lose their immunoreactivity and mRNA expression over a period lasting more than 2 months when gonadectomized. A comparison of the rates of loss in immunoreactivity and mRNA expression in the BNST was performed (Miller et al., 1992). Immunoreactivity following castration was significantly reduced at 3 and 8 weeks, but not at 1 week after castration. VP mRNA however declined much more rapidly, where there was a significant reduction in number of transcripts per cell and number of cells expressing VP mRNA by 1 week post castration, and there were no detectable cells expressing VP mRNA at 3 or 8 weeks after castration. The delay between mRNA and VP immunoreactivity in the BNST may be due to a reduction in the firing rates of VP neurons and of the rate of degradation of VP itself.

In situ hybridization using both the mature transcript probe and an intron probe to detect primary transcript, were also used in the Brattleboro rats to determine if the rates of transcription of the VP gene, albeit mutant, is the same for both the homozygous (HO) and heterozygous (HE) BB rats and the wild-type Long–Evans animals (Szot and Dorsa, 1992). The number of cells expressing VP mRNA in the supraoptic and paraventricular nuclei was not different between controls and the HO and HE BB rats, although the relative amounts of mutant transcript present per cell was significantly reduced in the HO rats. In contrast, the suprachiasmatic nucleus, the BNST and the amygdala all showed a reduction in total number of cells expressing VP mRNA and a reduction in the amount of transcript present per cell in both the HO and HE rats compared to the controls. To determine if the cause of these reductions were due to a decrease in transcription, we used an intron probe to detect primary transcript which indicated that the rates of transcription between the HO and HE BB rats were comparable to those observed in the control animals. Therefore, the reduction in cytoplasmic mRNA in the BNST and medial amygdala of the HO rat may be due to instability of the mRNA rather than decreased transcription. A subsequent study investigated the role of testosterone in impaired expression of VP mRNA in the HO rat. Plasma T levels were not different between controls, HE and HO rats. Additionally, castration and T replacement which was found to regulate the expression of VP mRNA in control and HE animals in these brain regions, had no effect on HO rats.

Having shown that gonadal steroid hormones potently modulate the expression of VP in the BNST and the medial amygdala of wild type animals, the effects of other steroid hormones, namely as those from the adrenals, were also assessed with reference to modulation of the expression of the VP gene in VP synthesizing neurons. To this end, animals were adrenalectomized and replaced with dexamethasone (Urban et al., 1991). mRNA for VP was unaffected by adrenalectomy in either brain region. Furthermore,

treatment with dexamethasone significantly reduced both the number of synthesizing cells and the number of transcripts expressed per cell. Measurement of plasma testosterone levels indicated that dexamethasone treatment significantly lowered mean T levels when compared to controls. Replacement of adrenalectomized animals treated with dexamethasone with silastic capsules containing T to maintain plasma T levels, blocked the effect of DEX on VP mRNA expression, suggesting that the decrease observed in the previous experiment was secondary to suppression of T by dexamethasone, probably by acting to diminish LH release from the pituitary.

Is there a behavioral consequence of the sex difference and steroid dependence of VP gene expression? The Brattleboro rat provides a possible model in which to study the sexual dimorphism of behaviors which might be related to differences in expression of VP. The parental Long–Evans strain display a sexual dimorphism in the retention of a conditioned taste aversion, in a paradigm using a sucrose solution paired with injections of LiCl, which induces illness in the rats. Females were more rapid to drink the sucrose following pairing with LiCl than were males. However, homozygous and heterozygous BB rats showed no sexual dimorphism indicating that intact levels of VP are necessary to observe the expression of the sexually dimorphic aversion behavior (Brot et al., 1992).

VP mRNA also declines in the BNST with age of the rat. There are significantly fewer VP mRNA expressing cells in a 24-month-old animal than in a 3-month or 14-month-old animal (Dobie et al., 1991). Additionally, there is a decrease in the content of VP mRNA transcripts per cell in the 24-month-old animal, which corresponds to the well documented reduction in plasma testosterone levels. The 24-month-old group had the lowest circulating plasma T levels when compared to middle aged and young animals. This indicates that the age related decline in VP neurotransmission may be related to age-related declines in plasma levels of testosterone. Another study investigated whether replacement of testosterone was capable of restoring VP mRNA content in the cells of the BNST of aged rats (Dobie et al., 1992). Administration of T completely reversed the decline of VP mRNA observed during senescence, in a dose dependent manner.

Thus it can be seen from the wide variety of studies carried out in our laboratory and in others, that a major determinant of the level of the expression of the VP gene in BNST and MA neurons is the ambient level of circulating gonadal hormones. The expression of the VP peptide is sexually dimorphic. Adrenal hormones do not appear to play a role in modulating the expression of extrahypothalamic VP neurons, as they do in the hypothalamic nucleus, the paraventricular nucleus. As described earlier, their effects may be primarily post synaptic. Additionally, gonadal hormones appear to exert some of their effects on the VP synthesizing nuclei BNST and medial amygdala through a transcriptional mechanism.

To further provide insights into the molecular basis of mechanism for the transcriptional effects of testosterone, or its metabolites dihydrotestosterone and estrogen on the VP gene, we have examined their effects on transcription mediated by $5'$ flanking regions of the VP gene. To this end we have studied approximately 5 kb of upstream flanking DNA of the rat gene (kindly provided by Dr David Murphy, Oxford, UK). We have used two different regions of the VP promoter, the most proximal 1.1 kb piece of DNA, and also approximately 5 kb of $5'$ flanking DNA of the rat VP gene which includes the most proximal 1.1 kb fragment. These segments of DNA have been attached to a luciferase reporter gene, and transfected into the human neuroblastoma cell line, SK-N-SH. Both fragments of DNA are responsive to estrogen in this cell line, when co-transfected with the human estrogen receptor, however, the 5 kb insert exhibits a more robust response to estrogen (Fig. 6). This indicates that the potential sites in the VP promoter which may support transcriptional effects of estrogen reside in the more distal 4 kb of DNA. Additionally, we have sequenced a large portion of this most distal 4 kb of genomic DNA, and have noted the presence of several putative estrogen response elements (ERE) sites, which differ from the consensus sequence by one or two nucleotides, depending on the element. Current studies are designed to evaluate the relationship of these

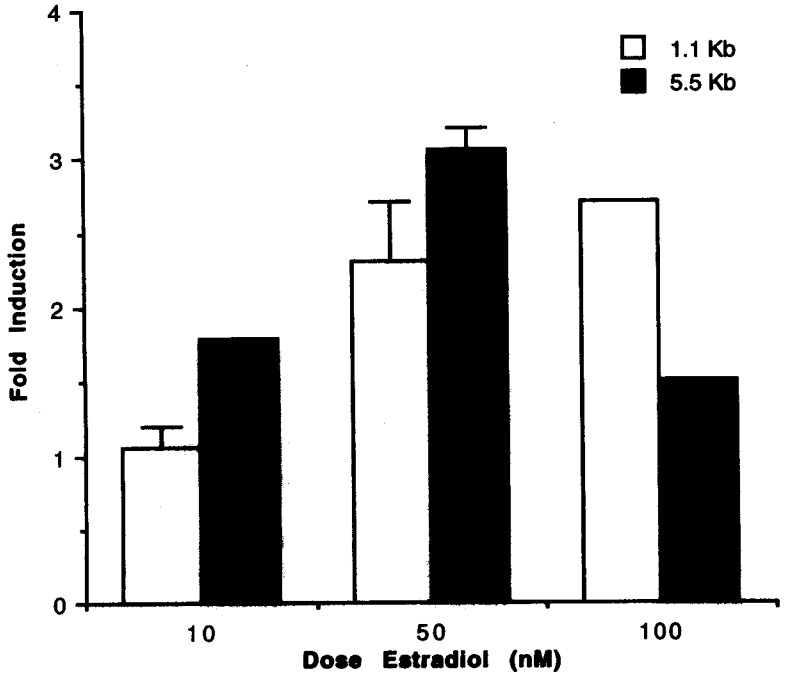

Fig. 6. Dose response curve of estrogen treatment on VP gene transcription in transiently transfected SK-N-SH cells. 1.1 kb (clear bars) and 5.5 kb (solid bars) fragments of the VP gene promoter.

observations in neuroblastoma cells to the transcriptional regulation we have evidenced in the rat brain.

Summary

In summary, we have provided considerable evidence both in vivo and in vitro that the transcriptional effects of steroid hormones on VP and VP receptor gene expression in brain represent a potentially important mode of modulation of the CNS effects of this neuropeptide system. While not yet entirely clear, we believe that the dependency of VP gene expression in extrahypothalamic neurons is related to behavioral differences between the sexes, including learning and memory. The influence of adrenal hormones on expression of V_{1a} receptors in the brain may be more important in modulating postsynaptic sensitivity to VP in stressful situations, perhaps leading to improvements in consolidation of memory. Such effects may have adaptive significance for the organism in encountering and avoiding situations which previously lead to the stressful event. Further studies will be required to substantiate these concepts.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 3.2.1

Vasopressin and oxytocin action in the brain: Cellular neurophysiological studies

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During the last two decades it has become apparent that vasopressin (VP) and oxytocin (OT), in addition to playing a role as peptide hormones, also act as neurotransmitters. Morphological studies and electrophysiological recordings have shown a close anatomical correlation between the presence of these receptors and the neuronal responsiveness to VP or OT. These compounds have been found to affect membrane excitability in neurons located in the hippocampus, hypothalamus, lateral septum, brainstem, spinal cord and superior cervical ganglion. Sharp electrode intracellular and whole-cell recordings, done in brainstem motoneurons, have revealed that VP and OT can directly affect neuronal excitability by opening non-specific cationic channels. These neuropeptides can also influence synaptic transmission, by acting either postsynaptically or upon presynaptic target neurons or axon terminals. Whereas in some hypothalamic neurons OT appears to mobilize intracellular calcium, as revealed by calcium imaging techniques, in the brainstem the action of this neuropeptide is mediated by a second messenger which is distinct from the second messenger activated in peripheral target cells. Future studies should be aimed at elucidating the properties of the cationic channels responsible for the neuronal action of VP and OT, at identifying the brain-specific second messengers activated by these neuropeptides and at determining whether endogenous VP and OT can exert neuronal effects similar to those elicited by exogenous neuropeptides.

Introduction

Vasopressin (VP) and oxytocin (OT) are peptide hormones which act on a variety of target organs, including kidney, smooth muscle, liver and anterior pituitary. During the last two decades it has become apparent that these two peptides may in addition act as neurotransmitters. A number of arguments supports this conjecture: (1) VP and OT are not only synthesized in hypothalamo-neurohypophysial cells, but also in other hypothalamic and extrahypothalamic cell bodies whose axon projects to the limbic system, the brainstem and the spinal cord; (2) VP and OT can be shed from central axons as are classical neurotransmitters; (3) central VP and OT may play a role in brain function, since in situ injection of VP and OT antagonists can interfere with behavior or physiological regulations; (4) specific binding sites, i.e., membrane receptors having high affinity for VP and OT are present in the central nervous system; (5) these receptors, or at least part of them, are localized on neurons, since application of exogenous VP and OT alters the rate of firing of single neurons present in regions where binding sites have been detected autoradiographically.

The neuronal actions of VP and OT have been investigated initially following microinjection and microiontophoresis in situ and more recently in vitro. Studies done up to the early 1990s are reviewed in Argiolas and Gessa (1991); De Kloet et al. (1990); De Wied et al. (1993); Dreifuss and Raggenbass (1993); Raggenbass et al. (1995) and Richard et al. (1991). Here we summarize more recent work. We concentrate upon studies carried

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out at the cellular level, i.e., restrict our review to in vitro systems. VP and OT are treated together. Indeed, although acting via distinct receptors in distinct brain areas, these two neuropeptides appear to exert similar effects upon neuronal excitability.

Mapping neuropeptide sensitivity within the brain

Light microscopic autoradiography has revealed the presence of V_{1a}-type VP and OT receptors in selected regions of the central and autonomic nervous system (for reviews, see Barberis and Tribollet, 1996; Zingg, 1996). Electrophysiological recordings, done mostly in brain slices of the rat, have shown a close anatomical correlation between the presence of these receptors and the neuronal responsiveness to VP or OT (for a possible exception, however, see the following section). As expected from the distribution of receptors, these neuropeptides have been found to affect excitability in neurons located in the hippocampus, hypothalamus, lateral septum, brainstem, spinal cord and superior cervical ganglion. New results have been recently obtained in the hypothalamus, brainstem and spinal cord.

Hypothalamus and limbic system

Studies in lactating rats suggest that OT may influence the milk-ejection reflex by acting in the bed nucleus of the stria terminalis (BST). Unit recordings, obtained from neurons located in the BST in hypothalamic slices from lactating rats, showed that about half of the neurons were excited by OT (Ingram et al., 1990). The proportion of responsive cells was unaltered during the peripartum period, but the sensitivity to the neuropeptide increased significantly during lactation (Ingram and Wakerley, 1993). In some OT-responsive neurons, orthodromic activation following hypothalamic electrical stimulation could be reversibly attenuated by an OT antagonist, suggesting the existence of an OTergic innervation of the BST (Ingram and Moos, 1992).

Neurons in the dorsomedial division of the suprachiasmatic nucleus (SCN) synthesize VP; in addition, VP mRNA transcription displays intrinsic rhythmicity. By acting within the SCN, VP may participate in the regulation of the circadian cycle. VP did indeed activate cells in the SCN in vitro. Interestingly, a V_{1a} antagonist reduced the spontaneous basal activity in VP-sensitive neurons, a fact suggestive of an endogenous excitatory VPergic input (Mihai et al., 1994a,b). However, the basal activity of VP-responsive neurons showed a marked circadian activity in both heterozygous (VP-containing) and homozygous (VP-deficient) Brattleboro rats, indicating that VP was not required for the generation of this circadian pattern of activity (Ingram et al., 1996).

In the ventromedial hypothalamus (VMH) of the rat, OT binding, as detected by autoradiography, is affected by modifications of the circulating gonadal steroid hormones. Castration drastically reduces this binding in animals of either sex, whereas gonadal steroid injection can restore it to normal levels. Accordingly, VMH neurons in hypothalamic slices from ovariectomized rats could be excited by OT, acting on OT receptors, and neuronal responsiveness increased following slice treatment with progesterone (Kow et al., 1991). However, the steroid dependence of OT receptors in the VMH appears to be species dependent, since in the guinea pig neither binding sites for OT nor neuronal responses to this neuropeptide were affected by gonadectomy (Inenaga et al., 1991; Tribollet et al., 1992).

Central administration of OT can depress prolactin secretion and part of this effect may be mediated by a neuropeptide-induced activation of tuberoinfundibular dopaminergic neurons. Consistent with this conjecture, OT, at nanomolar concentrations, could increase the excitability of a majority of dorsomedial arcuate neurons, which may include tuberoinfundibular neurons (Yuan and Pan, 1996).

In brain slices containing the subfornical organ (SFO), VP caused either an increase or a decrease in the discharge rate of angiotensinII-sensitive neurons. Whereas the excitatory effect was direct, the inhibitory effect was synaptically mediated and both were suppressed by a V_{1a} receptor antagonist (Anthes et al., 1997). Since the SFO is a brain region devoid of a blood-brain barrier, circulating VP may influence the activity of SFO neurons.

Functional V_{1a} receptors have also been detected

in neurons of the central amygdaloid nucleus. In this brain region, which contains a high level of VP binding sites, VP could indeed exert a direct excitatory action (Lu et al., 1997).

Electrophysiological recordings, performed in vivo and in vitro, indicate that VP and OT can alter the discharge rate of hypothalamic magnocellular neurons in the paraventricular and supraoptic nuclei (see, for example, Richard et al., 1991). In hypothalamic slices from virgin female rats, non-phasically firing neurons in these nuclei were inhibited by OT, whereas in slices from male or ovariectomized female animals, this neuropeptide had either no effect or caused excitation (Kuriyama et al., 1993). One should point out, however, that the presence of VP and OT receptors in the paraventricular and supraoptic nuclei is still an unresolved issue, different groups having reported conflicting results (Barberis and Tribollet, 1996). Thus, one cannot exclude that the electrophysiological effects cited above were exerted indirectly.

In lactating rats, OT supraoptic neurons respond to suckling by generating synchronous bursts of action potentials. In an attempt to elucidate the mechanism of this intermittent neuronal activation, intracellular recordings were done in hypothalamic organotypic slice cultures. In identified oxytocinergic neurons, the bursting activity, when present, was similar to that described in situ, though the interburst interval was much shorter. OT, acting on OT receptors, reduced the interburst interval in spontaneously bursting neurons and triggered bursting activity in some non-bursting neurons. Neither the spontaneous nor the OT-induced burst firing were due to intrinsic membrane properties of magnocellular neurons, but were dependent upon volleys of afferent excitatory postsynaptic potentials (EPSPs). This suggests that OT magnocellular neurons may be part of a hypothalamic rhythmic network, which is driven by glutamatergic synaptic transmission and which can be modulated by OT (Jourdain et al., 1998).

In acutely dissociated neurons from the horizontal limb of the diagonal band of Broca, VP caused a slight increase in an outward current (Easaw et al., 1997). This effect, which was detectable only at strongly depolarized potentials, was dependent upon extracellular calcium, was blocked by charybdotoxin and was mediated via V_{1a} receptors. In some neurons, however, VP caused a reduction, rather than an increase, in this outward current. This latter effect was suppressed by a V_2 receptor antagonist, an intriguing result in view of the fact that there is compelling evidence that brain VP receptors are of the V_{1a}-type.

Brainstem

In brainstem slices containing the dorsal vagal nucleus, a high proportion of neurons could be excited not only by OT, as found in our laboratory (see below), but also by VP, acting on distinct receptors (Mo et al., 1992). However, part of the recordings obtained by these authors may have been from adjacent solitary tract neurons, which are almost exclusively endowed with VP receptors (Raggenbass et al., 1989).

In the dorsal vagal complex, the neuronal sensitivity to OT was reduced following estradiol pretreatment of the animals, and was slightly enhanced by progesterone, suggesting that OT responsiveness in this brain region may change during the estrus cycle (Tolchard and Ingram, 1993). In this same preparation, a V_{1a} antagonist suppressed the response to VP but did not block completely the effect of vasotocin, providing suggestive evidence for a class of receptors distinct from either the OT or V_{1a} subtypes (Ingram and Tolchard, 1994).

Circulating VP is thought to modulate the sensitivity of the baroreceptor reflex by acting in the area postrema, which is located on the dorsal surface of the medulla and is outside the blood-brain barrier, whereas central VP may exert similar effects by modulating the activity of neurons in the nucleus of the solitary tract. Indeed, VP could affect the discharge rate of area postrema neurons in brainstem slices from the rat (Lowes et al., 1995) and the rabbit (Cai and Bishop, 1995). The neuropeptide action was mainly excitatory and, in the rat, it was at least in part direct and was mediated by VP receptors. In addition, by acting in area postrema (Cai et al., 1994), or upon vagal afferent neurons located in the nodose ganglion (Gao et

al., 1992), VP could indirectly influence the firing of solitary tract neurons.

Spinal cord

Intracellular recordings obtained from sympathetic preganglionic neurons in transverse slices of spinal cord indicated that VP and a V_{1a} agonist could cause cell depolarization, and that this effect was blocked by a V_{1a} antagonist (Sermasi and Coote, 1994). However, some sympathetic preganglionic neurons could be shown to possess functional OT receptors, whose activation increased membrane excitability (Desaulles et al., 1995). Thus, neurons located in the intermediate-lateral nucleus of the thoraco-lumbar spinal cord appear to be heterogeneous in their sensitivity to neurohypophysial peptides.

Membrane mechanism of neuropeptide action

The data reviewed so far suggest that VP and OT can affect the excitability of neurons located in various areas of the brain and spinal cord and may potentially subserve a variety of functions. However, with the exception of the studies done in hypothalamic organotypic slices and spinal cord, all these results were obtained using extracellular recordings and thus suffer from some limitations: the demonstration that the neuropeptide-induced effects are direct, rather than synaptically mediated, is difficult to obtain, the localization of the recorded neurons may be uncertain and no information can be deduced concerning the membrane mechanism of action of the neuropeptides.

More recently, the membrane mechanism of action of VP and OT started to be studied. To this end, one needs to get stable sharp electrode intracellular recordings from identifiable neurons responsive to these neuropeptides. We met such conditions by using brainstem slices containing the facial nucleus, the hypoglossal nucleus and the dorsal motor nucleus of the vagus nerve.

Facial nucleus

VP was found to increase the excitability of antidromically identified facial (VII) motoneurons (Tribollet et al., 1991). The membrane mechanism responsible for this effect was characterized using the single-electrode voltage-clamp technique. The neuropeptide acted by generating a persistent inward current, which was voltage-gated, tetrodotoxin-insensitive and sodium-dependent. Blockade of transmembrane calcium currents or partial substitution of chloride ions by isethionate did not significantly alter the VP-induced current. It was not affected by a two-fold decrease in the transmembrane potassium gradient and was not modified by a variety of potassium channel blockers (Raggenbass et al., 1991). Current-voltage relationships, obtained from cesium-loaded motoneurons, showed that the VP current reversed in polarity at around 0 mV, suggesting that it was a nonspecific cationic current. Reducing the extracellular calcium concentration caused a reversible increase in the amplitude of the VP current. Lowering the extracellular magnesium also increased this current, but less efficiently. These data indicate that divalent cations can modulate the VP response and suggest that in the normal physiological solution, which contains 2 mM calcium and 1 mM magnesium, the VP current is partially blocked (Alberi et al., 1993).

By inducing a persistent voltage-dependent inward current, vasopressin can affect the input-output relationship of facial motoneurons. Indeed, by using whole-cell recordings in the current-clamp configuration, we have recently assessed that in the presence of the peptide the current input required to attain the firing threshold was decreased and the frequency–current relationship was shifted to the left. This suggests that descending vasopressinergic pathways of hypothalamic origin may modulate the motor output by enhancing brainstem motoneurons excitability.

In transgenic mice overexpressing the protein Bcl2, axotomy-induced neuronal death of neonatal facial motoneurons is prevented, as assessed by morphological criteria. However, the functional properties of these surviving motoneurons are unknown. To clarify this issue, we have carried out whole-cell patch clamp recordings in brainstem slices of mice containing the facial nucleus (Alberi et al., 1996). We found that axotomized motoneurons in transgenic animals had properties similar to

those of intact motoneurons. They fired repetitively following positive current injection and, under voltage clamp conditions, they responded to ionotropic glutamate receptor agonists (cf. Widmer et al., 1992) as well as to VP by generating sustained inward currents. However, cell input resistance was much higher in axotomized motoneurons, indicating that they were smaller in size – an observation which was consistent with the morphological data. Thus, lesioned surviving facial motoneurons in transgenic mice appear to be endowed with functional receptors to neurotransmitters/neuromodulators.

Hypoglossal nucleus

Autoradiography revealed the presence of VP receptors in the ventromedial and dorsal division of the hypoglossal (XII) nucleus and VP was found to generate a sustained inward current in a majority of hypoglossal neurons. Antidromic activation, following electrical stimulation of nerve XII axons, or morphological characterization of biocytin-labelled neurons, indicated that at least part of the VP-sensitive cells were motoneurons. When synaptic transmission was blocked by perfusing the slice with a low-calcium/high-magnesium solution, the average peak amplitude of the VP-induced current decreased by 65%. Following tetrodotoxin treatment, this current decreased by a similar extent. In contrast, in a low-calcium/normal-magnesium concentration, i.e., in conditions of reduced synaptic transmission but of increased neuronal excitability, the VP current was not significantly altered. Thus, the action of VP was probably in part direct and in part presynaptic and the latter effect was dependent upon action potential propagation. Current–voltage relationships indicated that the inward current responsible for the postsynaptic effect reversed in polarity at around −15 mV, suggesting that it was carried by both sodium and potassium ions (Palouzier-Paulignan et al., 1994).

Dorsal motor nucleus of the vagus nerve

The dorsal motor nucleus of the vagus nerve (X) in the rat contains OT binding sites (Dreifuss et al., 1988). In early experiments it was found that OT could increase the excitability of a large proportion of neurons located in this nucleus (Charpak et al., 1984). Using morphological and electrophysiological criteria, some of the OT-responsive vagal neurons could be identified as being parasympathetic preganglionic motoneurons (Raggenbass et al., 1987; Tribollet et al., 1989; Dubois-Dauphin et al., 1992). The mechanism of action of OT was investigated in voltage-clamped vagal motoneurons. OT evoked a tetrodotoxin-insensitive, non-inactivating inward current whose peak amplitude was concentration-related. The OT current–voltage curve contained a region of negative slope conductance. Partial replacement of extracellular sodium reversibly attenuated or suppressed the neuropeptide current, whereas substitution of extracellular chloride or blockade of calcium currents did not modify it. Neither a decrease in the transmembrane potassium gradient nor any of several potassium channel blockers affected the OT current. Lowering the extracellular calcium concentration caused a reversible enhancement of the response to OT (Raggenbass and Dreifuss, 1992). These results indicate that OT excites vagal motoneurons by inducing a sustained voltage-gated inward current which is sodium-dependent and is modulated by calcium.

Characterization of neuropeptide-activated second messengers

Peripheral VP and OT receptors have been recently cloned in a variety of species. They belong to the G protein-coupled receptor family and possess seven hydrophobic transmembrane segments, connected by alternating extracellular and intracellular loops (for reviews, see Barberis and Tribollet, 1996; Zingg, 1996). In addition, the second messengers activated by VP and OT in peripheral target cells have been well characterized: while V_{1a} and OT receptors are coupled to a phospholipase C-β (PLC-β), V_2 receptors are linked to an adenylyl cyclase. By contrast, central receptors for VP and OT have not yet been cloned and the second messenger(s) involved in the central action of these peptides is (are) not yet known.

Recently, we have begun to characterize this messenger by using whole-cell recordings in brainstem slices containing OT-sensitive vagal neurons.

When loaded with GTP-γ-S, a non-hydrolyzable analogue of GTP, vagal neurons generated a persistent inward current in the absence of agonist; however, the OT effect was suppressed, suggesting that the neuropeptide-evoked current was mediated by G protein activation (Fig. 1). Loading vagal neurons with the calcium chelator, BAPTA, suppressed a calcium-dependent slowly decaying potassium aftercurrent, I_{AHP}, but did not affect the OT response, suggesting that the latter was not mediated by an agonist-induced increase in the intracellular free calcium concentration. Protein kinase C (PKC) activation was probably not involved in the neuronal effect of OT, since the neuropeptide-evoked current was not modified by loading neurons with PKC inhibitors (Raggenbass et al., 1995; Alberi et al., 1997). Thus, OT receptors in vagal neurons are probably not functionally coupled to a PLC-β. We are presently investigating whether at least part of the OT response may be mediated by a cAMP-dependent intracellular pathway.

Calcium imaging studies have recently revealed that in cultured rat supraoptic cells, VP and OT induced a significant increase in the intracellular calcium concentration. While the OT effect was exclusively due to calcium released from thapsigargin-sensitive stores, the VP effect required calcium influx from the extracellular medium, mainly through L-, N- and T-type calcium channels (Lambert et al., 1994; Dayanithi et al., 1996; Sabatier et al., 1997). VP- and OT-induced calcium transients have also been observed in cells derived from the organum vasculosum of the lamina terminalis and the subfornical organ (Jurzak et al., 1995) and VP, as well as angiotensin II, could increase the intracellular free calcium concentration in cultured neurons from the area postrema (Consolim-Colombo et al., 1996). In both systems, the neuropeptide effect persisted in the absence of extracellular calcium.

Neuropeptide modulation of synaptic transmission

In addition to directly affect the membrane ionic permeability in selected neuronal populations, VP

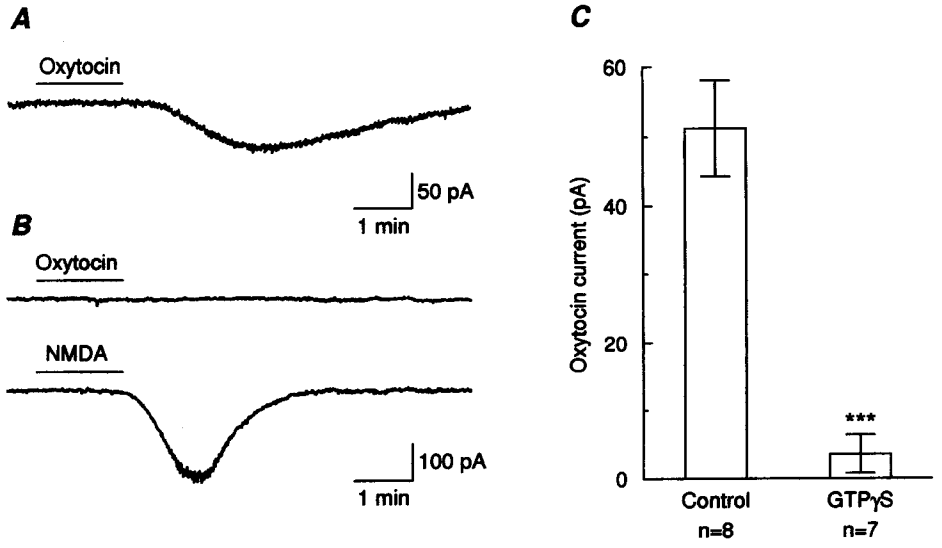

Fig. 1. The inward current generated by OT in vagal neurons is mediated by G protein activation. The left panel shows whole-cell voltage clamp recordings of membrane currents obtained in a control neuron (A) and in a neuron loaded with GTP-γ-S (B). Compounds were added to the perfusion solution for the 1.5-min period represented by the horizontal bar above each trace. The OT concentration was 0.2 μM in (A) and 1 μM in (B); the NMDA concentration was 50 μM. Note, in (B), that a persistent activation of G protein suppressed the OT but not the NMDA current. (C) Average inward current evoked by OT, at 1 μM, in control neurons and in GTP-γ-S-loaded neurons. ***$P < 0.001$.

and OT can influence neurotransmission at some central synapses.

We have recently uncovered an indirect effect of VP in the hypoglossal nucleus, using whole-cell recordings. Under voltage clamp conditions, VP caused an increase in the frequency of spontaneous postsynaptic currents (PSCs) in a majority of the recorded neurons. These neuropeptide-sensitive PSCs were negative in polarity in neurons held at or near their resting membrane potential and reversed in polarity at around −50 mV, a value close to the chloride reversal potential (Fig. 2). In addition, in chloride-loaded neurons, these PSCs were amplified and were inward-going at all membrane potentials. The stimulatory effect of VP persisted in the presence of CNQX, MK-801 and bicuculline, suggesting that neither AMPA/kainate nor NMDA nor $GABA_A$ receptors were involved. By contrast, it was suppressed by strychnine. We conjecture that, in addition to directly depolarize hypoglossal motoneurons (see above), VP may facilitate inhibitory synaptic transmission in the hypoglossal nucleus by acting upon the soma and/or axon terminals of putative glycinergic interneurons.

In hypothalamic supraoptic neurons, OT reduced the amplitude of inhibitory postsynaptic currents (IPSCs) mediated by $GABA_A$ receptors (Brussaard et al., 1996); this effect was probably due to a neuropeptide-induced increase in intracellular calcium, which in turn depressed inhibitory synaptic transmission via a postsynaptic mechanism. These results suggest that magnocellular neurosecretory neurons may be endowed with functional OT receptors (but see the section on the hypothalamus and limbic system) and that OT may control the activity of these neurons by disinhibiting them.

OT was also found to reduce the amplitude of excitatory postsynaptic currents (EPSCs) evoked in supraoptic neurons following electrical stimulation of afferent fibers. A similar reduction in excitatory input was observed following high frequency stimulation of afferents as well as by depolarizing single neurons by current injection. Since all these effects could be suppressed by an OT antagonist, it was suggested that dendritically released peptides can reduce excitatory synaptic input to magnocellular neurons by acting on presynaptic receptors (Kombian et al., 1997).

A modulatory effect of OT upon glutamatergic synaptic transmission has been evidenced in cultured neonatal spinal cord dorsal horn neurons (Jo et al., 1998). OT, or a selective OT agonist, caused a reversible increase in the frequency, but

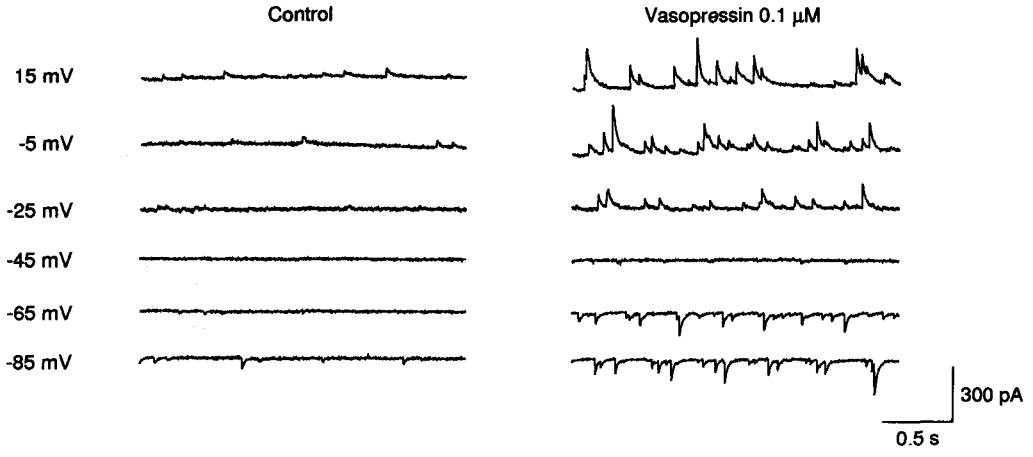

Fig. 2. VP enhances inhibitory postsynaptic currents (IPSCs) in hypoglossal motoneurons. The traces are current records, obtained in the whole-cell configuration, at holding potentials ranging from −85 to +15 mV. Currents were measured either in the absence (left panel) or in the presence of 0.1 μM VP (right panel). Patch pipettes contained a low-chloride solution. Note that the VP-enhanced IPSCs reversed in polarity at around −45 mV, i.e., close to the chloride reversal potential.

not the amplitude, of AMPA-receptor-mediated spontaneous, miniature and EPSCs. This peptide action was presynaptic and was dependent upon extracellular calcium influx. It might represent a neuronal mechanism by which descending hypothalamo-spinal OTergic pathways modulate sensory, and in particular nociceptive, input.

VP (Van den Hooff and Urban, 1990) as well as the C-terminal glycopeptide of the VP precursor (Van den Hooff et al., 1990) could slightly increase the amplitude of glutamate-mediated EPSPs elicited by stimulation of the fimbria axons. A slight, long-lasting enhancement of excitatory synaptic transmission could also be induced by VP and VP 4–8 in the CA1 field in hippocampal slices (Chepkova et al., 1995; Rong et al., 1993). These data suggest that VP, as well as some related neuropeptides, could influence synaptic plasticity. However, the pharmacological basis of this action is unclear, since the facilitatory effect of VP in the lateral septum was not suppressed by a V_{1a} receptor antagonist and it is not known whether the neuropeptide site of action was pre- or postsynaptic.

Interestingly, VP was found to enhance synaptic transmission at the frog neuromuscular junction. The neuropeptide acted presynaptically, by producing a long-lasting increase in spontaneous and evoked transmitter release; no postsynaptic effect could be detected at the frog neuromuscular junction (Abdul-Ghani et al., 1990).

Conclusions and perspectives

Extracellular recordings have been useful in mapping the VP and OT sensitivity in the central and peripheral nervous system and in showing that at least part of the binding sites having high affinity for these neuropeptides represent functional receptors located on neurons. The use of more advanced methods (intracellular and whole-cell recordings, calcium imaging) has allowed workers to begin to unravel the mode of action of these neuropeptides at a more mechanistic level. Some features emerge from these studies: (1) VP and OT can directly modulate neuronal excitability by opening nonspecific cationic channels, mainly permeable to sodium (facial, hypoglossal and vagal neurons; Delmas et al., 1997). (2) The neuronal second messengers activated by VP-and OT-receptor binding may be different from the second messengers stimulated by agonist-receptor interaction in peripheral target cells (cf. vagal versus supraoptic neurons). (3) VP and OT can exert powerful indirect effects by acting either postsynaptically (supraoptic nucleus) or upon presynaptic target neurons (hypoglossal nucleus).

In spite of some advances, however, a number of basic questions remain open. (1) The properties of the cationic channels responsible of the excitatory effects of VP and OT need to be further explored, possibly down to the single-channel level. To this end, cultured neurons, retaining their responsiveness to VP and OT, are needed. (2) The second messengers mediating the neuronal action of VP and OT need to be extensively characterized. Besides the whole-cell approach, microfluorimetric imaging of second messengers (calcium, cAMP, etc.), carried out in brain slices or in neuronal cultures, would be useful. In this context, it would also be important to determine how, besides exerting short term electrophysiological effects, VP and OT can have long term actions upon gene expression, thus influencing cell growth or morphology. (3) The demonstration that endogenous VP and OT can exert postsynaptic effects similar to those elicited by exogenously applied neuropeptides is still lacking. Dual recordings, carried out under visual control, in brain nuclei containing VP- and OT-synthesizing neurons as well as high affinity binding sites for these peptides may be helpful in this respect.

VP-deficient Brattleboro rats are viable, though they behave abnormally (Bohus and De Wied, this volume). However, they are viable for studies. In addition, two recent studies present evidence that mice lacking a functional gene coding for OT are viable and fertile, and do not present any major reproductive behavioral or functional anomaly. The only functional defect was the inability of the OT-deficient females to nurse their offspring (Nishimori et al., 1996; Young et al., 1996). These data, however, do not rule out a role for VP and OT in the brain. The existence of a great number of neurotransmitter systems suggests a high degree of functional redundancy in central signaling pathways. Thus, in VP- or OT-deficient

animals, alternative pathways may be called in action and substitute for the defective one.

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CHAPTER 3.2.2

Vasopressin acting at V_1-type receptors produces membrane depolarization in neonatal rat spinal lateral column neurons

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Vasopressin-immunoreactive fibers have been visualized in the area of spinal lateral horn cells, including spinal sympathetic preganglionic neurons. The presence and nature of vasopressin receptors on neurons in this area were addressed using whole-cell patch-clamp techniques in transverse spinal cord slice preparations from neonatal rat. Bath applications of Arg^8-vasopressin (VP) induced a slow-onset membrane depolarization accompanied by spike discharges and membrane oscillations. In voltage-clamp, applications of VP induced a reversible, tetrodotoxin-resistant and dose-dependent inward current in 90% of tested cells. This effect was blocked by a V_1 receptor antagonist [D-$(CH_2)_5$ Tyr (Me)-VP], whereas a V_2 receptor agonist [desamino-(D-Arg^8)-vasopressin] was ineffective. Furthermore the applications of oxytocin produced significantly smaller depolarizations when compared with VP suggesting that, at least in the neonatal lateral horn cells, vasopressin rather than oxytocin is more effective ligand. Both the amplitude and duration of the VP effect were enhanced after intracellular dialysis with GTP-γ-S, a non-hydrolyzable GTP analogue, whereas the inward current was significantly reduced after intracellular dialysis with GDP-β-S, a stable analogue of GDP that competitively inhibits G-proteins. The observation that the VP-induced net inward current reversed at a potential close to the equilibrium for potassium ions and was associated with a decrease in membrane conductance in a majority of tested cells suggest mediation through closure of a leak potassium conductance. These data indicate that SPNs and other lateral horn cells possess functional G-protein-coupled V_1-type vasopressin receptors that, in adult spinal cord, may contribute to CNS regulation of autonomic nervous system function.

1. Introduction

The nonapeptide $arginine^8$-vasopressin (VP) was one of the first neuropeptides to be isolated, sequenced and synthesized (du Vigneaud et al., 1954a). VP is commonly recognized as a hypothalamic neurohypophysial peptide that is synthesized in magnocellular neurons of the supraoptic and paraventricular nuclei and released from their posterior pituitary axon terminals to function as a circulating pressor agent and antidiuretic hormone (Acher, 1993). The observation that neurohypophysial peptides also influence behavior (see other chapters in this volume) and that neurons and axons demonstrating immunoreactivity for neurohypophysial peptides were detected in CNS regions unrelated to posterior pituitary control has fostered the notion that VP and oxytocin have a role in central neurotransmission (Buijs, 1987). Indeed application of VP alters the excitability of neurons in a variety of CNS sites (Muhlethaler et al., 1982; Ma and Dun, 1985; Raggenbass et al., 1988, 1991; Sun and Guyenet, 1989; Mo et al., 1992; Palouzier-Paulignan et al., 1994; Lowes et al., 1995). VP receptors belong to the family of G-protein coupled receptors, and functional studies conducted subsequent to the cloning and sequencing of VP receptor subtypes (Lolait et al., 1992, 1995; Morel et al., 1992; Barberis and Tribollet, 1996) have identified different roles for these receptors. Thus, circulating VP acts as an antidiuretic hormone at V_2-type

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receptors in kidney (Jard et al., 1975; Handler and Orloff, 1981) and a vasoconstrictor at arteriolar V_1-type receptors (Hofbauer et al., 1984). In brain, the diverse functions of VP in memory and behavior (De Wied et al., 1991), fever (Wilkinson and Kasting, 1987) and cardio-vascular regulation (Noszczyk et al., 1993) appear to be mediated by VP acting in a neuromodulator or neurotransmitter at V_1-type receptors.

In brain, several studies have now suggested that the parvocellular neurons located in the hypothalamic paraventricular nucleus (pPVN) are a source for the VP-immunoreactive fibers that are seen at brainstem and spinal levels (Sawchenko and Swanson, 1982). Anterograde tracers suggest that pPVN neurons project to the superficial layers of the spinal dorsal horn, and to the intermediolateral cell column, or IML, where they appear to innervate sympathetic preganglionic neurons, or SPNs (Saper et al., 1976; Hosoya et al., 1991). Immunohistochemical studies showing that a part of the spinal projections from pPVN are immunoreactive for vasopressin and oxytocin, (Swanson, 1977; Swanson and McKellar, 1979; Sawchenko and Swanson, 1982; Sofroniew, 1985; Cechetto and Saper, 1988) and observations that PVN lesions deplete levels of immunoreactive-vasopressin and -oxytocin throughout the spinal cord (Millan et al., 1984) confirm the supraspinal and hypothalamic origin of these spinal peptidergic projections. A role in autonomic and hydromineral regulation is suggested by data indicating an upregulation of mRNA for these peptides in spinal-projecting pPVN neurons in response to salt loading (Pretel and Piekut, 1989) and by the ability of an intrathecally-administered vasopressin antagonist to block renal nerve activity induced by electrical stimulation in PVN (Malpas and Coote, 1994). Thus, as suggested by the anatomical data (Hosoya et al., 1991) likely targets are VP-immunoreactive fibers from pPVN and VP receptors on SPNs.

Earlier electrophysiological observations do indeed indicate that VP acts at V_1 subtype receptors to depolarize spinal lateral horn neurons, including SPNs (Ma and Dun, 1985). In order to obtain more details on the mechanisms of action of VP on lateral horn cells, we have used in-vitro slice preparations and whole-cell patch-clamp techniques (Pickering et al., 1991; Kolaj et al., 1995) to examine VP-induced actions in SPNs and lateral horn neurons in the neonatal rat.

Vasopressin produces depolarization in spinal cord

Whole-cell recordings obtained from neurons located within the intermediolateral horn at the thoracolumbar level included SPNs, identified either antidromically following electrical stimulation of their axons in the ventral root exit site, or after injection of Lucifer Yellow, displaying typical oval or fusiform somata with characteristic dendrites extending medially towards the central canal (Shen and Dun, 1990; Pickering et al., 1991). No differences were observed either in membrane properties or in responses to drugs between identified SPNs and unidentified lateral horn cells, and therefore the data from these two groups of cells are considered together.

VP was generally applied by bath perfusion, but also by focal pressure ejection; since both methods yielded similar responses, these were analyzed together. As illustrated in the current-clamp recording in Fig. 1, a 1-min application of 1 μM VP was typically followed by a slowly-rising membrane depolarization that, upon reaching threshold, elicited action potential discharges, and required more than 10 min to return to resting membrane potential. This response to VP (i.e., membrane depolarization) reflects a direct action on postsynaptic receptors since it persisted in low-calcium high-magnesium ACSF and after blockage of action potential-dependent synaptic transmission with TTX. In SPNs, the response to 1 μM VP was often accompanied by subthreshold oscillations in membrane potential; the later feature has already been reported in SPNs (Shen et al., 1994; Logan et al., 1996).

In the absence of TTX, an increase in baseline thickening during the peak drug action was considered indicative of indirect or presynaptic actions, and was not analyzed further. It has been reported that spinal astrocytes also respond to VP (Hosli et al., 1991). However our recordings from several glial cells did not yield any indication of VP receptors.

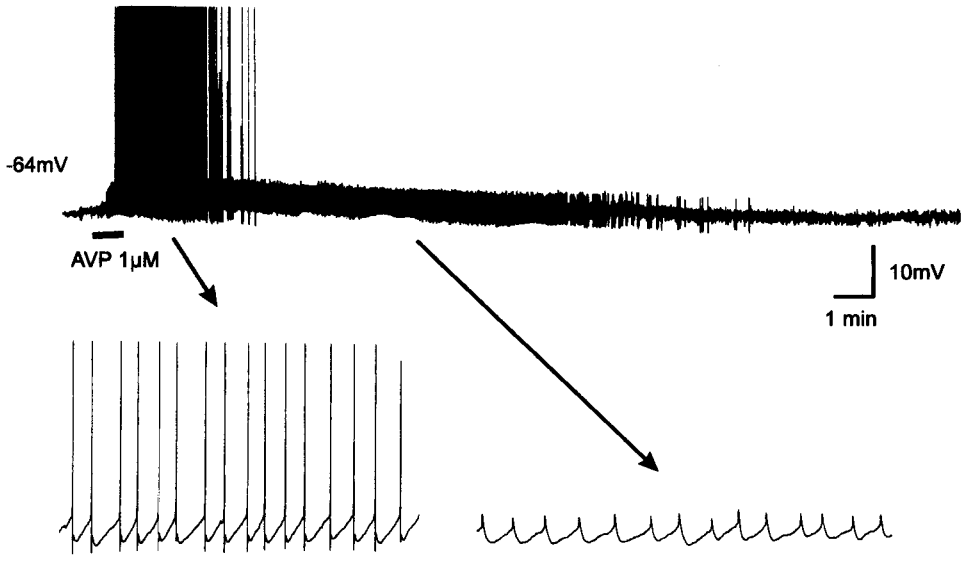

Fig. 1. Bath application of VP depolarizes lateral horn cells. In whole-cell current-clamp recording at a resting membrane potential of −64 mV, bath application of 1 μM VP for 40 s produces a slow-onset membrane depolarization accompanied by spike discharges and membrane oscillations.

These observations confirm the impression of a generally excitatory role for VP on neurons in neonatal spinal lateral horn (cf. Ma and Dun, 1985), hypoglossal nucleus (Palouzier-Paulignan et al., 1994), facial nucleus (Raggenbass et al., 1991), area postrema (Lowes et al., 1995), dorsal motor nucleus of the vagus (Mo et al., 1992), rostroventrolateral reticular nucleus (Sun and Guyenet, 1989), septum (Raggenbass et al., 1988) and hippocampus (Muhlethaler et al., 1982).

The mechanism(s) underlying VP-induced changes in cell excitability

The mechanism(s) underlying VP-induced changes in cell excitability were further examined under voltage-clamp conditions. Pressure application of VP (0.01–1 μM) induced reversible inward currents in 90% of tested cells (n = 100) held in −65 mV. Responses were slow in onset, reaching a peak in about 60 s, and slowly recovering towards resting values within 5–15 min (Fig. 2A). Conductance changes and the ionic basis of the slow depolarizing response evoked by 1 μM VP were examined with a voltage ramp protocol applied before and during the VP-induced response (Fig. 2A). Subtracting the ramp obtained at the peak of the VP response from that obtained prior to application of VP yielded the net VP current (Fig. 2B). While this could reveal any voltage sensitivity of the current suppressed by VP, these plots indicated that this net VP current was not particularly voltage-sensitive in that its amplitude was linearly related to the driving force upon K^+ ($V_m - E_{K+}$; Fig. 2B). The linearity of this current assures that it will contribute substantially to the 'leak' conductance of the membrane potential, even at rest. Furthermore, this analysis in 21 cells also revealed a 16.4 ± 2.6% decrease in input conductance during the VP-induced response, indicated by the reduced slope of the ramp. In contrast, 4 cells displayed an increase in membrane conductances to 116.2 ± 1.9% of control, while the remaining cells showed no change in membrane conductances. Within the testing voltage range (−40 to −120 mV), the reversal potential of the net VP in 20 cells was −105.9 ± 4.1 mV. These findings suggest a K^+ conductance as the principal source of the VP-induced inward current since (a) the net VP current reversed at a reversal potential close to

the estimated K^+ equilibrium potential (in our experimental conditions −98.6 mV), (b) the net current decreased at hyperpolarizing levels presumably due to the reduced driving force for K^+ ions at potentials closer to E_{K+}, (c) net current was associated with a decrease in apparent membrane conductance in a majority of tested cells. This conclusion was further substantiated in 3 neurons where a reversal potential of −104 ± 4 mV under control conditions with 3.1 mM $[K^+]_{out}$ changed to −70 ± 3 mV in media containing 10 mM $[K^+]_{out}$ as predicted by the Nernst equation for a K^+ current. These results provide convincing evidence that reduction of a resting (so called 'leak') K^+ conductance contributes to the VP-induced inward current in SPNs. In the rest of the tested cells, control and test ramps showed a parallel shift with practically no change in membrane conductance.

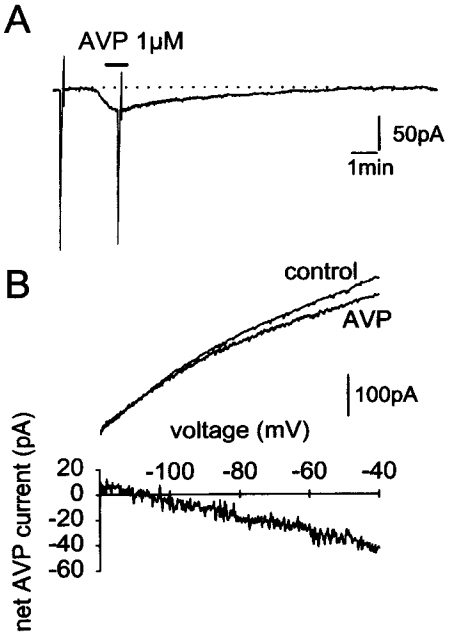

Fig. 2. VP induces a slow inward current that is associated with a decrease in a resting potassium conductance. (A) In this cell, under voltage-clamp conditions at -65 mV and in the presence of 0.5 μM TTX, application of VP induced a slow rising, prolonged and reversible inward current. (B) The ramp analysis (voltage is linearly increasing (8 s) from −120 to −40 mV) reveals that VP decreases the membrane conductance with a reversal potential close to the potassium equilibrium potential. The net VP-induced current was constructed by subtraction of the ramp obtained at the peak of the VP response (VP) from that obtained prior to application of VP (control). Notice the linearity of this net current.

The voltage clamp analysis provides new insight into the mechanisms whereby VP produces membrane depolarization of these spinal neurons. In a majority of these cells, application of VP induces an inward current and reduction in a resting potassium conductance; voltage ramp tests reveal that this current has a linear *I–V* relationship. The characteristics of the potassium current modulated by VP result in a specific type of increased excitability. Since this current contributes to the resting membrane potential, VP can facilitate initial responsiveness to excitatory neurotransmission by depolarization as well as by increasing input resistance and by increasing synaptic efficiency towards the somas when compared with dendrites. The so-called 'leak' resting potassium conductance is present in many mammalian neurons, and is a target for neurotransmitters and/or neuromodulators to effect a change in neuronal excitability. For example, this conductance can be blocked by activation of α_1 adrenergic (Elliott and Wallis, 1992; Larkman and Kelly, 1992), H_1 histaminergic (McCormick and Williamson, 1991), muscarinic (Guerineau et al., 1994), 5HT serotonergic (Elliott and Wallis, 1992; Larkman and Kelly, 1992), metabotropic glutamatergic (Guerineau et al., 1994), substance P (Fisher and Nistri, 1993), and TRH (Fisher and Nistri, 1993) receptors.

Vasopressin effect is mediated through activation of specific V_1-type receptors

As mentioned earlier, vasopressin actions are mediated through either V_1 or V_2-type receptors. In lateral horn cells, most vasopressin receptors appear to be of the V_1-type. Cells were tested for a response to VP before and after bath application of [D-$(CH_2)_5$Tyr(Me)-VP], a specific V_1-type receptor blocker (Manning compound; Barberis and Tribollet, 1996). While not affecting membrane conductance itself, application of this antagonist (0.1–1 μM) for 10 min effectively and reversibly prevented the VP-induced membrane depolariza-

tion and/or inward current (Fig. 3). Further support for mediation via V_1-type receptors was the failure of a DDVP, a V_2 receptor agonist, to evoke comparable responses to those observed with VP, even at 10-fold concentrations (not illustrated). Using whole-cell patch-clamp recording techniques, the studies reported here confirm that bath or pressure applied VP has a postsynaptic membrane depolarizing action and increases the excitability of a majority of neurons in the neonatal rat spinal lateral column, including identified SPNs, through activation of specific V_1-type receptors.

Vasopressin response is dominant over the response to oxytocin

Results from earlier studies with neurohypophysial peptides suggested a possible cross talk between receptors responding to VP and to a structurally related peptide, oxytocin (du Vigneaud et al., 1954b). In neonatal rat lateral column neurons, applications of oxytocin (1–2 μM for 60 s) produced significantly smaller depolarizations when compared with 1 μM VP (3 ± 0.8 mV versus 15.4 ± 3.9 mV; $n = 5$; $P < 0.05$) suggesting that, at least in the neonatal lateral horn cells, vasopressin rather than oxytocin is the ligand for the effects observed in the present analysis (cf. Sermasi and Coote, 1994). However, it has also been shown (Desaulles et al., 1995) that oxytocin could induce larger effect through activation of specific oxytocin-receptors. The discrepancy between these studies could be due to the fact that the intermediolateral nucleus, where most of these recordings were performed, shows a high anatomical heterogenity, and different subnuclei may be distinguished by distinct projections from PVN, particularly so for the oxytocinergic projections (Appel and Elde, 1988). Although the present data indicate that neonatal lateral horn cells do respond to oxytocin, the response is clearly smaller and involves a smaller proportion of cells. This is interesting since data from immunocytochemical studies in adult rat would suggest a preponderance of oxytocin fibers over vasopressin fibers in spinal cord (Sawchenko and Swanson, 1982). The issue of developmental change in these receptors needs to be addressed.

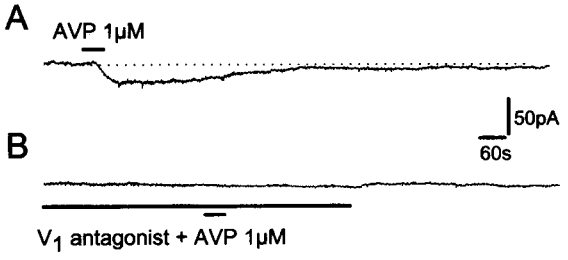

Fig. 3. V_1-type receptors mediate VP responses. VP application produces an inward current in this neuron recorded with the nystatin perforated patch-clamp technique. In whole-cell voltage-clamp recording in the presence of TTX (0.5 μM), pressure application of VP (1 μM for 45 s) produces slow reversible inward current (A) that is blocked by pretreatment with a V_1 receptor specific antagonist (B; Manning compound, 0.1 μM for 15 min).

Vasopressin action requires activation of G-proteins

A concern with whole cell patch clamp technique is that cell dialysis may alter or obscure drug-induced responses. Therefore a perforated-patch technique was used to verify the VP-induced responses. Similar to the experiments carried out using conventional whole-cell recordings, applications of 1 μM VP produced membrane depolarization and an inward current (−38.7 ± 11.3 pA; $n = 8$) that was indistinguishable from the results described above, indicating that intracellular dialysis did not alter the VP-evoked responses (Fig. 3).

Vasopressin receptors belong to the family of G-protein coupled receptors (Lolait et al., 1995; Barberis and Tribollet, 1996). An approach to testing the involvement of G-proteins in the intracellular transduction pathways involves the intracellular introduction of non-hydrolyzable analogues of GDP and GTP (Gilman, 1984). Interestingly, the mere inclusion of GTP in the pipette solution resulted in a 25% increase in the magnitude of VP-induced inward current (Fig. 4B). Inclusion of GTP-γ-S, a non-hydrolyzable derivative of GTP that activates G-proteins in an irreversible manner, caused a further increase of VP response by more than 100% (Fig. 4B). Whereas the response to VP normally recovered within a few minutes under

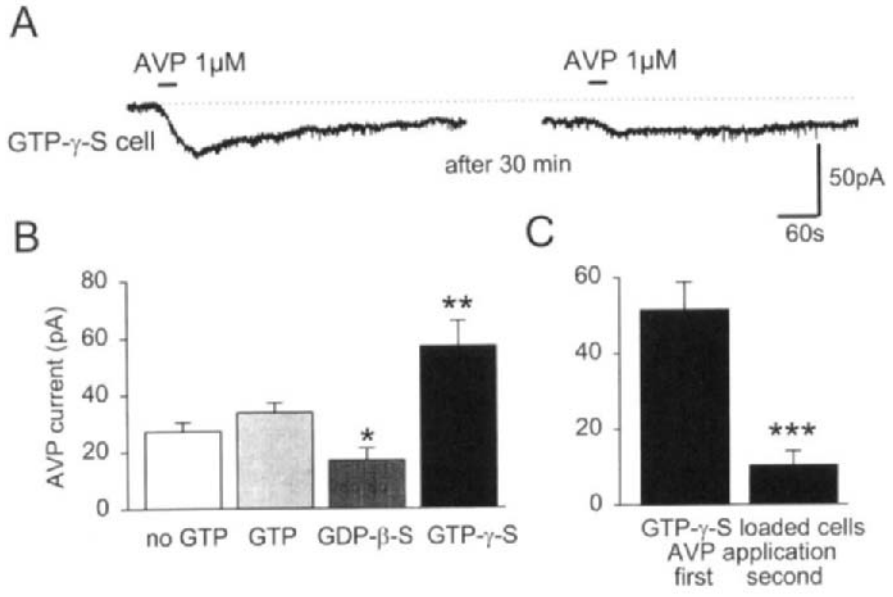

Fig. 4. G-proteins are involved in the VP-induced responses. (A) Patch clamp recording with a pipette containing GTP-γ-S (0.2 mM; for 10 min) irreversibly enhances the current produced by 1 μM VP (for 30 s). VP response does not recover and response to a second VP application after 30 min produces significantly smaller response (C; $n = 8$). (B) Summary data of the maximal inward current produced by 1 μM VP in cells not receiving GTP ($n = 39$) and by 1 μM VP in cells pretreated either with GTP ($n = 56$) or with GDP-β-S ($n = 10$) or with GTP-γ-S ($n = 16$). All analogues of GDP or GTP were applied intracellularly through the patch pipette solution for at least 10 min. Data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, as compared to values obtained without pretreatment (B) or to values obtained during first VP application (C).

control conditions (Fig. 2A), recovery in cells dialyzed with GTP-γ-S was not evident up to 35 min after drug wash (Fig. 4A). Thus the response to VP appeared to be virtually 'irreversible', with the response to a second application yielding significantly smaller effects (Fig. 4A,C). Finally, GTP-γ-S produced a gradual inward current about −15 pA in magnitude in 45% of tested cells. In another experiment, cells were exposed to intracellular GDP-β-S, a stable analogue of GDP which competitively inhibits G-protein binding of, and activation by, GTP. The presence of GDP-β-S in the cell interior would therefore be expected to inhibit a G-protein-mediated effect of VP. Cells were dialyzed for >10 min before application of VP. Diffusion of GDP-β-S into the cells significantly reduced the inward current induced by VP (Fig. 4B) when compared to cells treated with GTP. These observations strongly support the involvement of G-proteins in the VP-induced responses and confirm the prediction from molecular studies that VP receptors belong to the family of G-protein coupled receptors (Lolait et al., 1995; Barberis and Tribollet, 1996).

Although assumed to exist, no electrophysiological studies have thus far identified possible second messenger systems that might participate in the response to VP. The slow onset and prolonged duration of the VP effect suggest a complex signal transduction mechanism. In other systems, it is known that V_1 subtype receptors can activate phospholipase C with subsequent production of IP_3 and DAG, which will turn on protein kinase C and promote an intracellular release of calcium (Creba et al., 1983; Berridge, 1987). Recently it was shown that VP could increase intracellular calcium in primary cell cultures of organum vasculosum of the lamina terminalis and subfornical organ (Jurzak et al., 1995). It remains to be determined if a similar phenomenon occurs in lateral horn neurons. It is

noteworthy that all receptors which can block resting potassium conductance will also activate phospholipase C and IP_3 production (Fisher et al., 1992) but at the moment there is no clear evidence that this is related to gating of an inward current via blocking potassium channels. Quite possibly the inward current is gated through a direct interaction between a G-protein subunit and the potassium channel, a mechanism that has been proposed for cardiac muscarinic-gated potassium channels (Wickman and Clapham, 1995).

Comparison with VP effect on other tissues

Interestingly, VP appears capable of affecting neuronal excitability through mechanisms that are different from those seen in lateral horn neurons. For example, in brainstem motoneurons, VP produces a TTX-resistant inward current through opening of persistent voltage-dependent sodium channels (Raggenbass et al., 1991; Palouzier-Paulignan et al., 1994). Reasons for such differences in mechanisms of response may reflect receptor coupling through the same, or different, G-protein(s) that in turn influence different conductances, and these may be tissue specific and developmentally dependent. Pharmacological data indicate that V_1-type VP receptors can modulate different conductances in different tissues. Thus, in hepatocytes VP increases $[Na^+]_{in}$ and activates cation-selective channels, which likely account for VP- activated calcium influx (Lidofsky et al., 1993). In guinea-pig ventricular myocytes, and in insulin-secreting cells, VP causes a potentiation of voltage-sensitive calcium (probably L-type) channels (Zhang et al., 1995; Thorn and Petersen, 1991). Even more complex is the VP action on A7r5 rat smooth muscle cells where V_1-type receptor activation stimulates IP_3 formation (Thibonnier et al., 1991) and mobilization of intracellular calcium stores with consequent activation of capacitative calcium entry. In addition, VP also activates bivalent cation entry and a calcium efflux pathway (Byron and Taylor, 1995). The released of calcium from intracellular stores is connected with transient activation of Ca^{2+} sensitive K^+ conductance and inhibition of L-type of calcium channels through PKC activation, where both these effects are related to vasopressin induced hyperpolarization. In

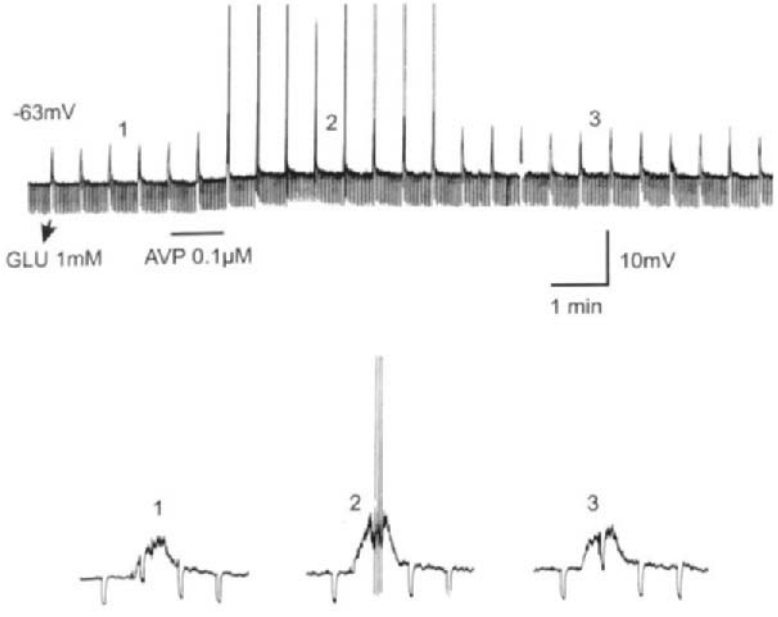

Fig. 5. VP increases membrane excitability to glutamate. Repetitive pressure application of glutamate (GLU; 1 mM for 1 s) induces transient membrane depolarizations that are potentiated during co-administration of 0.1 μM VP (for 1 min). Note the minimal depolarization with this concentration of VP.

addition, vasopressin provokes the activation of inward current due to opening of non-selective cation channels (Van Renterghem et al., 1988).

Physiological consequences of depolarizing action of vasopressin

Membrane depolarization by VP is likely to enhance neuronal excitability to exogenously applied excitatory neurotransmitters such as glutamate. Indeed, in experiments using regular pressure applications of glutamate to elicit a consistent subthreshold membrane depolarization, a marked increase in the glutamate-evoked responses was observed after the addition of a low concentration of VP (0.1 μM for 45 s) that produced only a small membrane depolarization and minimal change in membrane resistance (Fig. 5).

Do these VP receptors on spinal lateral horn cells have a physiological function? Most likely yes. In-vivo intrathecal administration of VP produces cardiovascular and antidiuretic responses that can be blocked by a receptor antagonist (Riphagen and Pittman, 1985a,b), and intrathecal administration of an VP receptor antagonist can block renal nerve activity produced by stimulation of PVN (Riphagen and Pittman, 1989; Malpas and Coote, 1994). VP receptors in spinal cord may also have some neurotrophic role based on data by Tribollet et al. (1994) that demonstrate an increased number of VP binding sites at lumbar spinal cord 14 days after axotomy, and by Iwasaki et al. (1991) who noted a neurotrophic action of VP, but not oxytocin, in explanted spinal cord cultures. Perhaps a trophic function is one explained for the high proportion of VP-responsive neurons in neonatal spinal cord.

In summary, the present data indicate that SPNs and other spinal lateral horn cells possess functional V_1-type VP receptors that are G-protein coupled, and produce membrane depolarization through reduction in a resting potassium conductance. This action may contribute to an excitatory role of spinal vasopressin in modulating autonomic functions.

Acknowledgements

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 3.2.3

Effects of vasopressin and related peptides on neurons of the rat lateral septum and ventral hippocampus

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The effects of vasopressin (VP), VP fragments and propressophysin glycopeptide on neuronal activities in the septum-hippocampus complex of rats were studied in vitro and in vivo. The frequency of the hippocampus theta rhythm in Brattleboro rats homozygous for diabetes insipidus was significantly slower than that of heterozygous litter mates and normal rats. Intracerebroventricular micro-injection of des-glycine-amide vasopressin corrected for several hours the frequency deficit of the theta rhythm in the homozygous Brattleboro rats and the centrally administered VP slowed down theta rhythm in normal rats. Microinotophoretically administered VP excited single neurons in the lateral septum of ventral hippocampus, and/or facilitated the responses of these neurons to glutamate and to stimulation of the glutamatergic afferent fibers in the fimbria bundle. The excitatory effects of VP vanished within seconds after termination of the peptide administration, however, the peptide-induced enhancement of glutamate and syntatically induced excitations were sustained for up to 60 min after the peptide administration. In vitro, pM concentrations of VP, VP 4–8 and C-terminus glycopeptide of propresophysin facilitated for 30–60 min the glutamate-mediated EPSPs in neurons of the lateral septum or the ventral hippocampus. The EPSPs increase in the lateral septum neurons was not prevented by pretreatment with antagonist of the V_{1a} type of the vasopressin receptor. The resting membrane potential and input resistance were not affected by the peptides. A low-frequency electrical stimulation in the diagonal Band of Broca or in the Bed nucleus of the stria terminals, sources of the vasopressinergic innervation of the septum, facilitated the negative wave of the filed potentials responses evoked in the lateral septum by stimulating the fimbria bundle fibers in control Long–Evans and Brattleboro rats heterozygous for diabetes insipidus. The field potential increase was sustained for several hours after the stimulation, and it was not occluded by long-term potentiation elicited by high frequency stimulation of the fimbria bundle afferent fibers. Brattleboro rats homozygous for diabetes insipidus failed to show the filed potential increase after the diagonal band stimulation. It is suggested that the long-lasting facilitation of glutamate-mediated excitations might be a physiological action of the propressophysin-derived peptides in the septum-hippocampus complex which, in concert with other forms of synaptic plasticity like the long-term potentiation, facilitates the hippocampus-mediated forms of learning and memory. This action is presumably related to the memory enhancing effect of the propressophysin-derived peptides.

Introduction

The lateral septum and CA_1/subiculum of the ventral hippocampus are among the sites in the brain in which vasopressin (VP)-containing fibers and specific binding sites for these hormones have been found (for review, see Brinton; De Vries, Du et al.; Tribolet et al., this volume). Also synaptically mediated release of VP in the septum, hippocampus and in other brain areas has been demonstrated (Buijs and Heerikhuize, 1982;VPDisturnal et al., 1984; Demotes-Mainard et al., 1986; Naylor et al., 1988; Neumann et al., 1988; Ramirez et al., 1990; Landgraf et al., this volume). Centrally administered VP improves avoidance behaviour, stimulates the maintenance of tolerance to alcohol, and affects the regulation of body temperature and of many other physiological parameters (for review, see Kovács and De Wied, 1994; see also Albers and Bamshad; Brinton; Dantzer; Jurzak and

Schmidt; Landgraf et al.; Popik et al; Reimers et al.; Sarnai; Smock et al.; Wakerley et al.; Wang et al. in this volume). Learning of a passive avoidance response and retrieval or relearning of a Go-No Go discrimination task is markedly improved by picogram quantities of VP or VP 4–9 injected into the lateral septum or ventral hippocampus of rats or mice (Kovács et al., 1979, 1986; Metzenger et al., 1993), indicating that the peptides have a powerful influence on the functioning of these structures. Further, it appears that the brain metabolizes VP into shorter peptides, such as VP 4–9 or [pGlu^4,-Cyt^6]VP 4–8, which are even more potent than VP to improve the learning of a passive avoidance response (Burbach et al., 1983; De Wied et al., 1984), but which lack the pressor and/or antidiuretic activity of the parent hormone (De Jong et al., 1985). On the basis of these and other findings (De Wied et al., 1989), it has been suggested that brain VP and VP metabolites may function as memory-facilitating principles (De Wied, 1991). The beneficial effect of centrally administered VP and VP 4–8 on learning of passive avoidance responses was demonstrated when the peptide was administered 60 min prior to learning (De Wied, 1971), or immediately thereafter (Kovács et al., 1986), but also when the learning trial was given 3 h after VP administration (Gaffori and De Wied, 1986). Thus, the memory enhancing effect of VP and VP 4–8 is presumably the result of a long-lasting action of the peptides, which modulates excitability of the brain neurons rather than directly activating these neurons. The typical feature of neuromodulation in this sense is that the peptide alone does not activate receptor-gated channels as do most of the classical neurotransmitters such as glutamate, GABA, acetylcholine, noradrenaline, dopamine and serotonin. Instead, it triggers via receptor-mediated mechanisms, intracellular processes that alter the functioning of one or more types of the receptor-channel complexes for minutes or hours. Activation of transmitter receptors by their ligands is the condition for the effect of the peptide to manifest itself. The well known facilitatory action of benzodiazepines on GABA-ergic hyperpolarization mediated by the $GABA_A$ receptor-channel complex (Study and Barker, 1981) was one of the first examples of such a modulatory action. In our research on the function of brain VP and related peptides we were particularly interested in examining whether small amounts of VP and its derivatives could modulate the excitability of the brain neurons for a period of time that would be comparable to the duration of effectiveness of the peptide in behavioural studies.

Effect of VP and related peptides on lateral septum and hippocampus neurons in vivo

In the first series of experiments we chose to study the effect of VP on the hippocampus theta rhythm in rats. This peculiar and very prominent electroencephalographic rhythm of the hippocampus, described in Green and Arduini (1954), was thought by some investigators (Landfield et al., 1970; Longo and Loizzo, 1973; Landfield, 1976) to be related to the memory function of this structure (for review, see Squire, 1992). The theta rhythm in rats accompanies several kinds of locomotor activity, and its frequency can vary between 4 and 12 Hz, depending on the velocity and/or intensity of movements. In view of feature it has been associated with, and implicated in, the regulation of voluntary and goal-directed movements (Vanderwolf, 1971). However, a very regular theta rhythm of a frequency of approximately 7.5 Hz occurs in the hippocampus during rapid eye movement (REM) sleep (Parmeggiani and Zanocco, 1963). This particular sleep stage was shown to be important for the process of memory consolidation (Epson and Clarke, 1970; Fishbein, 1971; Pearlman and Greenberg, 1973; Fishbein et al., 1974; Linden et al., 1975; Leconte et al., 1976). Rats of the Brattleboro strain, due to a one base deletion in the VP gene, are unable to synthesize brain VP (Schmale and Richter, 1984) and suffer from hereditary diabetes insipidus (DI) (Valtin, 1967). Brattleboro rats homozygous for DI (HO-DI) have difficulty maintaining conditioned avoidance behaviour, a difficulty which is readily corrected by a single systemic or intracerebroventricular injection of VP (De Wied et al., 1975), suggesting that VP or related peptides may be important for memory function in rats. We found no significant difference in the total amount of REM sleep in HO-DI rats and in homozygous

normal (HO-N) control rats, although the REM sleep episodes in HO-DI rats were slightly longer but less frequent than those of HO-N rats. The slow-wave sleep of HO-DI rats was slightly but significantly shorter than that of control rats. However, power spectral analysis of the EEG during REM sleep showed that the mean frequency of the theta rhythm during REM sleep episodes in H-DI rats is nearly 1 Hz (0.87) lower than that of HO-N rats. A single intracerebroventricular injection of 0.02 μg des-glycinamide^{9}-arginine^{8}-VP (DG-VP), a derivative of VP said to have negligible antidiuretic and pressor activities (De Wied et al., 1989), increases the mean theta frequency of the HO-DI rats to the level of that in the control HO-N rats, without significantly affecting the amounts of REM and SW sleep episodes. This DG-VP effect was maximal 60–120 min after peptide administration and vanished within the next 24 h. Specific antiserum raised against VP, injected in 1–2 μl quantity in one of the lateral cerebral ventricle, slows down the REM sleep theta rhythm frequency of HO-N rats to the frequency of that in H-DI animals (Urban and De Wied, 1975, 1978), suggesting that centrally secreted VP (Mens et al., 1982) might be of importance for normal functioning of the theta rhythm generating network. Later, it was found that some animals of the Brattleboro strain of HO-DI rats showed an average 38% deficit in the daily duration of the REM sleep (Danguir, 1983).

The theta rhythm activity in the hippocampus of rats originates from a phasic activity of the medial septum neurons which, in turn, is a result of tonic excitation of the septum conveyed to the septum network by the ascending activating system from the mesencephalic reticular formation (for review, see Vertes, 1982; Bland, 1986). Thus, the medial septum network functions as a pacemaker of the hippocampus theta rhythm (Petche et al., 1962). In the late 1970s, the brain VP system was discovered and was shown to innervate, among other structures, the lateral septum, amygdala, and ventral hippocampus (Buijs, 1978). VP-containing synapse-like contacts have been found in the septum and elsewhere in the brain (Buijs and Swaab, 1979). Also Ca^{2+}-dependent release of VP from the lateral septum (Buijs and Heerikhuize, 1982) and other structures was demonstrated (for review, see Landgraf et al. in this volume), suggesting that VP or a closely related neuropeptide may have a function in neuronal communication, either as a neurotransmitter or as a neuromodulator. In subsequent experiments we decided to inject minute amount of VP directly into the septum and examine the frequency of the REM sleep theta rhythm of thus treated rats. As little as 0.01 pg of VP dissolved in 1 μl of saline solution and injected slowly into the septum accelerated by approximately 0.5 Hz the frequency of the REM sleep theta rhythm for several hours, indicating that the septal neurons are very sensitive to this peptide. The frequency spectrum of SW sleep activity of the hippocampus was not affected by VP administered thus (Urban, 1981). Interestingly, 0.01 pg of oxytocin (OT) injected into the septum had an effect similar to that of VP and accelerated the REM sleep theta rhythm (Urban, 1981), an effect opposite to the slowing of the theta rhythm by OT when several-fold greater amounts of this peptide were injected into one of the cerebral ventricles (Bohus et al., 1978). Thus, minute amounts of VP could alter the functioning of the theta rhythm producing network in the septum for a number of hours, suggesting that this, or a similar effect of VP could be related to the memory enhancing, long-lasting action of the peptide.

In the next series of experiments we decided to examine the effect of VP on single neurons. We applied VP locally by microiontophoresis and recorded the spontaneous activity of the lateral septum neurons. Approximately 20–30% of the lateral septum neurons studied appeared to be briefly excited by thus applied VP. The VP induced excitations started and ended rapidly after the start and ending of the iontophoretic administration, an effect resembling the excitation of these neurons elicited with similarly applied glutamate (Joëls and Urban, 1982). A similar, short-latency, short-lasting action of iontophoretically applied VP also excited the neurons of other brain areas, including the hypothalamus, dorsal hippocampus, brainstem and spinal cord (Huwyler and Felix, 1980; Marchand and Hagino, 1982; Tiberiis et al., 1983; Backman and Henry, 1984; Thornton et al., 1985). In contrast, the rat cortical neurons were mostly inhibited by a locally applied, relatively high concentra-

tion of VP (Webb et al., 1986). The number of lateral septum neurons directly excited by iontophoretically applied VP was surprisingly small, amounting to approximately 30% of the neurons tested (Joëls and Urban, 1982). In fact, most of the lateral septum neurons showed no noticeable change in spontaneous activity during and after such application of the thus applied peptide. However, the excitations induced in these neurons with 5–10 s pulses of glutamate were markedly increased during peptide application. Surprisingly, the VP-induced increase in the glutamate responses lasted for ca. 10–60 min after termination of the peptide application (Joëls and Urban, 1984a).

The lateral septum complex receives, by way of the fimbria bundle, prominent innervation from the hippocampus, which is excitatory in nature (De France et al., 1973a,b), topographically organized within the lateral septum (Joëls and Urban, 1985a) and which uses glutamate as neurotransmitter (Storm-Mathisen and Woxen-Opsahl, 1978; Zacek et al., 1979; Walaas and Fonnum, 1980; Joëls and Urban, 1984b,c). The long-lasting facilitation by VP of the neuronal responses to glutamate suggested to us that the peptide might facilitate glutamate-mediated synaptic responses of lateral septum neurons in the same way as it facilitated the responses to glutamate. Indeed, nearly all neurons that showed an increase in glutamate responses also showed an increase in the number of action potentials in response to fimbria bundle stimulation. In many of the neurons studied, the responses to fimbria bundle stimulation remained increased for 10–60 min after termination of the peptide administration (Joëls and Urban, 1984a). The response of the lateral septum neurons to iontophoretically applied noradrenaline, serotonin or GABA was little affected by the peptide (Joëls and Urban, 1985b), suggesting that this long-lasting facilitatory action of VP might be specific to glutamate-mediated synaptic responses.

The hippocampus is another structure in which VP-immunoreactive fibers and binding sites for VP and OT have been found. The effects of microiontophoretically applied VP on CA1/subiculum neurons in the ventral hippocampus appeared to be very similar to effects in the lateral septum. Nearly all the neurons studied in the ventral hippocampus were briefly excited by glutamate or by acetylcholine, but only approximately 30% of these neurons were directly excited by a 10 s pulse of VP. The remaining 70% of the neurons showed no noticeable change in the spontaneous firing rate during VP application. However, the responses of these neurons to glutamate, but not to acetylcholine, were markedly increased during the application of the peptide, and the peptide-induced increase in response mostly persisted for several minutes to an hour. Both the VP-induced and the glutamate-induced excitations were prevented when an antagonist of the V_{1a} type of VP receptor, $d(CH_2)_5$-Tyr-(Me)-arginine^8-VP (Kruszynski et al., 1980), was co-released with VP or glutamate. Interestingly, the V_{1a} VP receptor antagonist failed to prevent the facilitatory action of VP on the glutamate-induced excitations (Urban and Killian, 1990). These results led us to suggest that VP may have two pharmacologically distinct actions on neurons in the ventral hippocampus. One, which directly excites the neurons and which can be blocked by V_{1a} receptor antagonists, and the other, which has no effect on spontaneous activity but which specifically facilitates the neuronal responses to glutamate and which is resistant to the action of the V_{1a} receptor blocker. However, the depression of the glutamate-induced excitation by the V_{1a} receptor antagonist caste doubt as to the specificity of this VP antagonist in the brain. Non-specific depressing actions of the V_{1a} receptor antagonist on spinal neurons were also demonstrated by others (Porter and Brody, 1986).

The enzymes present in a synaptosomal plasma membrane fraction cleave VP into several shorter peptides. Some of these peptides, such as VP 4–9 and VP 4–8, appeared to be even more potent than VP to facilitate the avoidance behaviour of rats (Burbach et al., 1983) but did not show measurable pressor and antidiuretic activities such as those of VP (De Jong et al., 1985). Only a small (approximately 25%) population of the ventral hippocampus neurons studied were directly excited by iontophoretically applied VP 4–9 and VP 4–8. The time course and magnitude of these excitations were very similar to those induced in the hippocampus neurons by the whole VP molecule or by glutamate. Most of the neurons tested showed no

noticeable change in firing rates during or after peptide application. However, the responses of the neurons to glutamate, shown to be a major excitatory transmitter in the hippocampus (for review, see Storm-Mathisen, 1977; Cotman et al., 1987; McDonald and Johnston, 1990), were markedly increased during peptide application and for more than 60 min afterward (unpublished observation).

Effects of VP related peptides on lateral septum and hippocampus neurons in vitro

Dreifuss and his co-workers (Mühlethaler et al., 1982) were the first to study the effect of VP in hippocampus slice preparations in vitro. Applied to the bath 10^{-6-8} M VP slightly depolarized presumed pyramidal neurons and markedly augmented the firing rate of these neurons. This excitatory action of VP was visible as early as several seconds after the start of application, usually vanished within 10 min after peptide application ended, could be elicited repeatedly and was blocked by d(CH_2)$_5$-Tyr-(Me)-arginine^8-VP ([1-(β-mercapto-β,β-cyclopentamethylpropionic acid)2-(*O*-methyl)tyrosine]arginine^8-VP; Kruszynski et al., 1980), an antagonist at the V_{1a} type of peripheral VP receptor. This suggested that VP could function as an excitatory neurotransmitter in the hippocampus. Subsequent studies showed that especially the presumed inhibitory interneurons in the CA1 field of the hippocampus were strongly excited by the bath applied VP. Simultaneous recording from two neurons showed that the firing rate of small-spike, interneuron-like neurons increased greatly during VP application whereas the firing of larger, presumably pyramidal, neurons was inhibited during peptide administration (Mühlethaler et al., 1984). A similar excitatory action of 10^{-6-8} M VP on neurons in the dorsal hippocampus was demonstrated by others (Mizuno et al., 1984), although this group considered the neurons excited by VP as pyramidal cells. In addition, VP was shown to excite the neurons in the lateral septum (Raggenbass et al., 1987a, 1988), dorsal cochlear nucleus (Charpak et al., 1989), nucleus of the rats solitary tract (Raggenbass et al., 1989b), the lateral horn cells of the neonatal rat spinal cord (Ma and Dun, 1985), guinea-pig inferior mesenteric ganglion cells (Peters and Kreulen, 1985) and newborn spinal cord neurons (Suzue et al., 1981). The V_{1a} receptor antagonist blocked the VP excitation in most of these studies. The facial nucleus of a new-born rat is rich in [^{3}H]VP binding sites that disappear later during ontogeny (Tribolett et al., 1991). A 30 s bath application of 1.0–0.1 μM VP elicited, in a voltage-clamped neonatal facial neuron, a tetrodotoxin-resistant, voltage-dependent inward current which ceased within 5 min following the end of the application. Partial replacement of Na^+in the medium by equimolar *N*-methyl-D-glucamine reduced the inward current, suggesting Na^+ as the ion mediating this current (Raggenbass et al., 1991, 1993). Low calcium/high magnesium conditions did not interfere with the effect of VP, suggesting a direct postsynaptic action of the peptide. The type of VP receptor mediating the inward current was not identified in these experiments. Whether this or a similar inward current also mediated the short-lasting excitations of neurons in adult hippocampus and in other brain structures remains to be investigated.

Interestingly, bath-applied 0.5–1.0 μM OT excited the pyramidal and non-pyramidal neurons in the CA1/subiculum of the rat hippocampus in the same way as did VP (Raggenbass et al., 1989a). OT also briefly excited neurons in the dorsal motor nucleus of the nervus vagus (Raggenbass et al., 1987b), inducing a sustained sodium-dependent current (Raggenbass and Dreifuss, 1992). The effect of OT and VP was also studied in sympathetic preganglionic neurons in neonatal rat spinal cord. Both peptide hormones excited the sympathetic neurons much as did the VP-induced inward sodium-mediated current in brainstem autonomic neurons. A peripheral OT receptor agonist failed to mimic this effect of OT, and an antagonist of the V_{1a} type of VP blocked both the OT- and VP-induced excitations (Sermasi and Coote, 1994), suggesting that the OT effect was mediated by V_{1a} type receptor for VP. Thus, the mechanism of the excitatory action of OT appeared to be similar to that for VP action. The potent excitatory action of OT on antidromically identified neurosecretory neurons in the paraventricular nucleus of the hypothalamus was known from earlier iontophoretic studies (Moss et al., 1972). OT also excites

neurons in the bed nucleus of the stria terminals (see Wakerley et al., this volume), and the brain neurons (for review, see Richard et al., 1991).

Electrical stimulation of the fimbria bundle, the major input system to the lateral septum from the hippocampal formation (Raisman, 1966; Swanson and Cowan, 1977), evokes in lateral septum neurons excitatory postsynaptic potentials (EPSPs) that are followed by fast and slow inhibitory postsynaptic potentials (IPSPs). The fast IPSPs originate from an action of GABA on the $GABA_A$ receptor as they can be blocked with picrotoxin or with bicuculine, specific antagonists at the $GABA_A$ receptor-channel complex. The slow IPSPs are a result of GABA action on $GABA_B$ receptors (for review, see Gallagher et al., 1995). We developed a brain slice preparation that contains a nearly entire dorsolateral septum nucleus with preserved input from the fimbria bundle (Urban, 1987a). Picrotoxin added to the lateral septum slice markedly increases the EPSPs in lateral septum neurons in response to fimbria bundle stimulation. The picrotoxin-induced EPSPs increase can be abolished by D-2-amino-5-phosphonovaleric acid (2PV) (Van den Hooff et al., 1989a), a specific antagonist of *N*-methyl-D-aspartate type of glutamate receptors (Watkins and Evans, 1981), confirming the presence of a NMDA receptor-mediated component in thus induced EPSPs in lateral septum cells that had been reported by others (Gallagher and Hasuo, 1989). Bath-administered 0.1 μM VP failed to directly excite most of the neurons tested in this preparation. In fact, induction by VP of spontaneous firing of neurons in the lateral septum slice was rare, and when it did occur, was associated with a slight, up to 8 mV depolarization. The resting input resistance of the membrane either did not change or increased as a result of peptide administration. However, some of the lateral septum cells exposed to this concentration of the peptide had a marked shortening of the delay to the first action potential in response to depolarizing current injection, indicating an effect of the peptide on the postsynaptic membrane. In addition, the peptide also increased the magnitude of the excitatory, glutamate-mediated EPSPs evoked in neurons by stimulation of the fimbria bundle without a noticeable change in the resting input resistance of the neurons. The direct membrane action was prevented by the addition of 1 Mμ of a V_{1a} receptor antagonist to the bath. The facilitation by VP of the action potential generation vanished within several min after washout of the peptide (Van den Hooff et al., 1990a), and thus resembled the direct excitations of the hippocampus and other brain neurons by VP and OT that were discussed above. Thus, at a micromolar concentration, VP can increase both, the excitability of the postsynaptic membrane of the lateral septum neurons and the magnitude of EPSPs in these neurons. The short duration of the former effect, and the doses need to elicit it suggested that this effect was unrelated to the memory-enhancing action of the peptide.

The long-lasting facilitating effect of VP on EPSPs in lateral septum neurons seemed interesting because this effect mostly outlasted the period of administration by nearly an hour. This resembled the duration of the effectiveness of VP like peptides to stimulate the maintenance of avoidance behaviour. In fact, in several lateral septum neurons that we could record for a period longer than 60 min, the VP-induced increase of EPSPs often lasted for the entire recording period. Therefore, we decided to examine the effect of the peptide on EPSPs in greater detail. Picrotoxin blockade was used to block the fast, $GABA_A$ receptor-mediated IPSPs which interfered with the EPSPs (Van den Hooff et al., 1989a). In addition, we chose to use pM concentrations of VP in the bath as these concentrations resembled most the doses of the centrally administered peptide used in the avoidance behaviour studies (Kovács et al., 1986). Approximately 55% of the lateral septum neurons studied showed an increase in EPSPs when as little as a 1 pM concentration of VP (10^{-12} M) was added to the medium for twenty min. The peptide-induced EPSPs increase ranged from 10 to 45% of the baseline level and was not associated with a noticeable change in the resting membrane potential and input resistance of the neurons. In approximately half of the neurons, the EPSPs increase disappeared within 15 min of the washout period although, in a few neurons EPSPs remained increased for the entire recording period (up to 30 min). While higher concentration of VP in the bath (10^{-10} M) failed to produce a greater effect, we did find the long-

lasting increase in EPSPs in a greater number of cells. Addition of 1.0 nM V1 receptor antagonist to the VP-containing medium failed to prevent the enhancing effect of 0.1 nM VP on EPSPs. Also, a NMDA glutamate receptors antagonist failed to prevent the peptide effect on EPSPs (Van den Hooff and Urban, 1990a), indicating that the NMDA component in the EPSPs was not affected by the peptide.

VP is synthesized as part of a larger precursor peptide, propresophysin, also containing neurophysin II and a C-terminus glycopeptide which is a sequence of 39 amino acids, CPP_{1-39} (Brownstein et al., 1980; Land et al., 1982). Cleavage of propresophysin presumably occurs in the synaptic vesicles prior to release, indicating that the glycopeptide and neurophysin II are co-released with VP. The C-terminus glycopeptide is present in all VP brain systems (Lu et al., 1982; Watson et al., 1982), and the presence of shorter CPP glycopeptides in the brain, CPP_{22-39} for example, was demonstrated (Segers and Burbach, 1987a,b). We studied in a series of intracellular experiments with lateral septum as a slice preparation the effect of 1 pM bath-applied CPP_{22-39}. A 20-min application of the glycopeptide potentiated EPSPs in lateral septum neurons in the same way as did the same concentration of VP. Neurophysin II, in up to a 100-fold higher concentration had no such effect (Van den Hooff et al., 1990b), suggesting that the released C-terminus glycopeptide and VP may facilitate each others action.

Several conclusions could be drawn from results of these experiments. First, very low concentrations of VP and C-terminus glycopeptide are able to induce long-lasting facilitation of glutamate-mediated EPSPs, indicating that neuronal processes mediating glutamatergic transmission in the lateral septum are extremely sensitive to VP and to glycopeptide. Second, the receptor type(s) involved in this effect of the two principles was presumably different from the V_1 type mediating the blood pressure increase by VP, and third, the VP-induced EPSPs increase was not due to facilitation of the NMDA glutamate receptor-mediated component of the EPSPs. The effect of CPP_{22-39} was not tested in the presence of NMDA receptor antagonists. Regarding the potentiating effect on EPSPs, VP's effect differed from the NMDA glutamate receptor-mediated long-term potentiation (LTP) of glutamate-mediated EPSPs, which was first described for the dentate gyrus of the hippocampus by Bliss and Lømo (Bliss and Lømo, 1973) and was later also found in the CA1 field of the hippocampus and in many other brain regions (for review, see Teyler and Di Scenna, 1987). This form of synaptic plasticity requires, among others, high-frequency activation (above 10 Hz; most frequently 50–100 Hz stimulation is used) of the postsynaptic NMDA glutamate receptor and associated calcium influx to the postsynaptic neurons (for review, see Bliss and Collingridge, 1993), although activation of the postsynaptic metabotropic glutamate receptors also seems to be essential for LTP induction (Bashir et al., 1993), and it is visible immediately after the termination of synaptic activation. LTP has been suggested as one of the mechanisms involved in the memory-encoding function of the hippocampus (for review, see Eichenbaum and Otto, 1992; Squire, 1992). Therefore, in another series of experiments with lateral septum slices, we examined whether endogenous VP plays a role in the induction and/or expression of LTP in the glutamatergic synapse of neurons of the lateral septum.

Role of VP in LTP in lateral septum

High-frequency stimulation (HFS) of the fimbria bundle at 50 Hz for 2 s elicits LTP in EPSPs in neurons of rat lateral septum as slice preparations (Van den Hooff et al., 1989b). Thus induced LTP was prevented by 2APV, a specific antagonist of NMDA glutamate receptor (Watkins and Evans, 1981), indicating that LTP in the glutamatergic synapse in the lateral septum is similar to that in the CA1 field of the hippocampus (for review, see Collingridge and Bliss, 1993). We used brain slices from Brattleboro rats heterozygous (HE) and homozygous (HO) for DI. The brain of Brattleboro HO-DI rats, due to a base deletion in the gene of propressophysin (Schmale and Richter, 1984), contains no VP and CPP (Vandesande and Dierickx, 1976; Glick and Brownstein et al., 1980; Horn et al.,

1985; Dubois-Dauphin and Zakarian, 1987; Van Leeuwen et al., 1989). Indeed, with stimulation at 50 Hz for 2 s we were able to induce a LTP-like increase in EPSPs or in the negative wave of the filled potentials (FPs), that was elicited by fimbria bundle stimulation, in nearly all the slices from HE-DI rats. In slices from Brattleboro HO-DI rats, the same stimulation of the fimbria bundle elicited only an initial EPSPs or FPs increase which decayed to the baseline level within 30–45 min after the stimulation, indicating that LTP was not induced in slices from DI rats. Either perfusion of slices from HO-DI rats for 2 h with a medium containing 100 pM VP prior to the administration of HFS, or administering VP to the rats for 1 week prior to experiments with slices, corrected the LTP induction defect in slices from HO-DI Brattleboro rats (Van den Hooff et al., 1989b). However, blockade of $GABA_A$ receptor-mediated inhibition by picrotoxin in the bath also corrected the deficit in LTP induction in slices from HO-DI rats (unpublished observation). This showed that NMDA receptor-coupled cellular mechanisms mediating LTP induction in lateral septum are present and functioning in HO-DI Brattleboro rats, although the activation of these mechanisms is impaired in the absence of endogenous VP. The manner in which VP facilitates LTP expression in the lateral septum of HO-DI rats was not elucidated in these experiments. However, it is conceivable that the augmenting effect of VP on the size of EPSPs might have been at least partly, responsible for correction of the impairment of activation mechanisms of LTP induction in HO-DI Brattleboro rats. Interestingly, a normal LTP could be induced in the CA1 field of the hippocampus in slices from HO-DI Brattleboro rats (unpublished observation), indicating that VP or related peptides are not required for the LTP form of synaptic plasticity in this region of the hippocampus. Whether this also is the case for the LTP induction in the dentate gyrus is not known. Also, it remains to be investigated whether the behaviorally active shorter metabolites of VP, VP 4–9 or VP 4–8, are needed for the expression and maintenance of LTP in the rat lateral septum-hippocampus complex.

Effects of VP and related peptides on neurons in the ventral hippocampus in vitro

It is generally recognized that the hippocampus plays a key role in elaborating spatial and other forms of memory (for review, see Eichenbaum and Otto, 1992; Squire, 1992; McLelland et al., 1995). VP, VP 4–9 or VP 4–8 microinjected into the hippocampus facilitates avoidance behavior of thus treated rats (for review, see Kovács and de Wied, 1994), indicating that facilitation of the avoidance behavior presumably resulted from peptide-induced alteration of hippocampus function. Recently, we studied the effect of pM concentrations of VP and VP 4–8 on neurons of the CA1/subiculum field of the rat ventral hippocampus in slice preparation (Chepkova et al., 1995). Addition of 10^{-10} M VP to the bath increased EPSPs evoked in the hippocampus neurons by electrical stimulation of the afferent fibers in the stratum radiatum as had been seen earlier for lateral septum neurons. A rise in the amplitude and the slope of EPSPs was mostly visible at the end of the 20 min exposure to VP, attaining a maximum 30–50 min from the start of the application. EPSPs started to decline during the 60 min washout period in approximately 35% of the neurons studied. However, in most of the cells the increase in slope and amplitude of the EPSPs lasted unchanged for the entire 60 min of the recording (see example in Fig. 1A). In some neurons, the VP-induced EPSPs increase was sufficient to initiate action potentials on the EPSPs (see example in Fig. 1B). In these cases, the first action potential appeared at the beginning of the rising phase of the EPSPs and was often followed by one or more spikes (see example in Fig. 1B). As in the lateral septum neurons, potentiation of EPSPs by VP occurred without measurable changes in the resting potential and input resistance of the membrane. The stimulus-response relation EPSPs increased after VP application in a manner reminiscent of that in LTP experiments (Andersen et al., 1980; Racin et al., 1983); removal by picrotoxin of the $GABA_A$ receptor mediated inhibition did not interfere with the facilitating effect of the peptide on EPSPs. Block of NMDA type of glutamate receptors by 2APV did not prevent the VP-induced EPSPs increase, indicating that the NMDA recep-

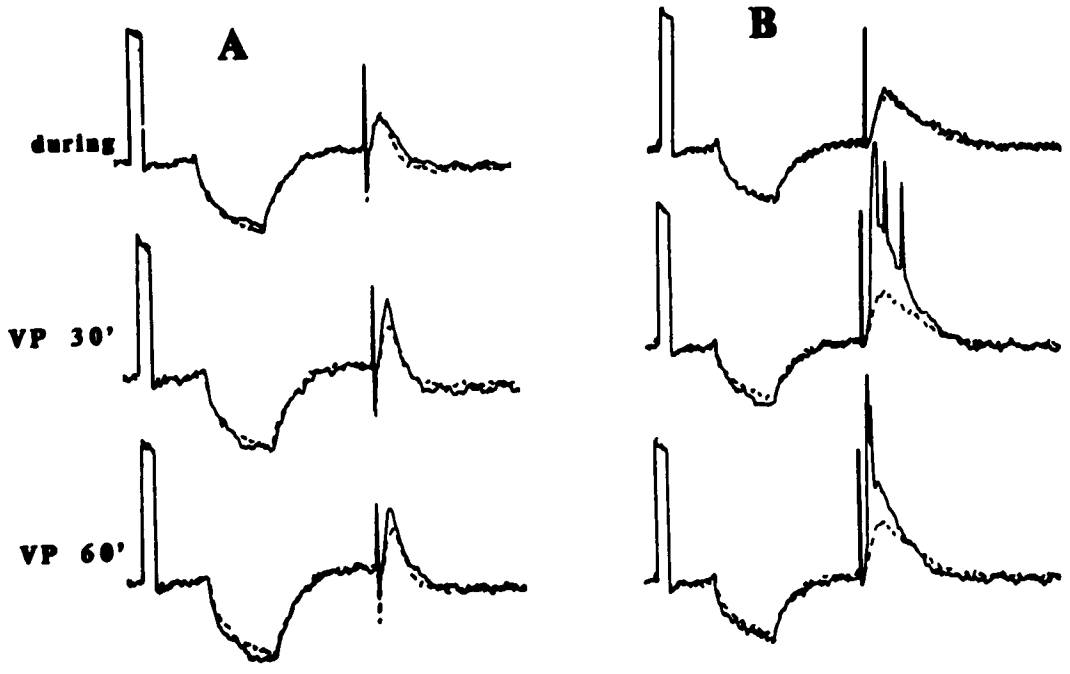

Fig. 1. Example of average EPSPs ($n = 10$) in two CA_1/subiculum neurons (A and B), recorded with KAc-filled electrode (85 MΩ resistance), during the baseline (con), 20-min bath application of 0.1 nM VP (15 min), and at 30 and 60 min after the start of peptide administration. The action potentials superimposed on EPSPs in neuron B were truncated by averaging. The resting membrane potential of neurons A and B was −68 and −63 mV, respectively. The hyperpolarizing current in A and B was 0.2 and 0.1 nA, respectively. The responses to this current indicated no marked change in the input resistance of the neurons in the course of the experiment. Calibration: 10 mV; 5 ms (reproduced with permission from Chepkova et al., 1995).

tor-mediated component of EPSPs in the hippocampus neurons (Hestrin et al., 1990; for review, see Collingridge and Lester, 1989) was not affected by the peptide. The effect of 10^{-10} M VP 4–8 was very similar to that of VP. This peptide also augmented the amplitude and slope of the EPSPs for more than 60 min after its wash-out. The VP 4–8-induced EPSPs increase was often associated with firing of multiple action potentials, like that seen after VP application. The resting membrane potential and input resistance were not changed by VP 4–8 (see examples in Fig. 2). Concentrations of VP 4–8 lower than 10^{-10} M were not tested in our experiments. These results confirm the results of Du et al. (1994), who reported a long-lasting potentiation by VP and VP 4–8 of EPSPs in neurons of the CA1 field of the dorsal hippocampus in vitro (Rong et al., 1993). Glutamate-mediated EPSPs in CA3 neurons were potentiated by VP and VP 4–8 in the same way as the CA1 neurons. The potency of VP 4–8 to facilitate EPSPs in these experiments was approximately a thousand-fold greater than that of the whole VP molecule (for review, see Du et al., this volume). This was comparable to the potency ratio of this fragment for facilitation of avoidance behavior (Burbach et al., 1983). Others (see Brinton, this volume) have reported long-lasting potentiation by nanomolar VP of the field EPSPs in the CA1 field of the hippocampus. This effect was seen only when extracellular calcium was lowered to 1.5 mM. Interestingly, 2.5 mM calcium in the bath reversed the potentiation into a lasting depression of field EPSPs (Chen et al., 1993). The effect of shorter VP peptides under these conditions was not studied.

The site of action, receptor type and intracellular effector mechanisms mediating the effects of VP and VP 4–8 on EPSPs have not yet been identified.

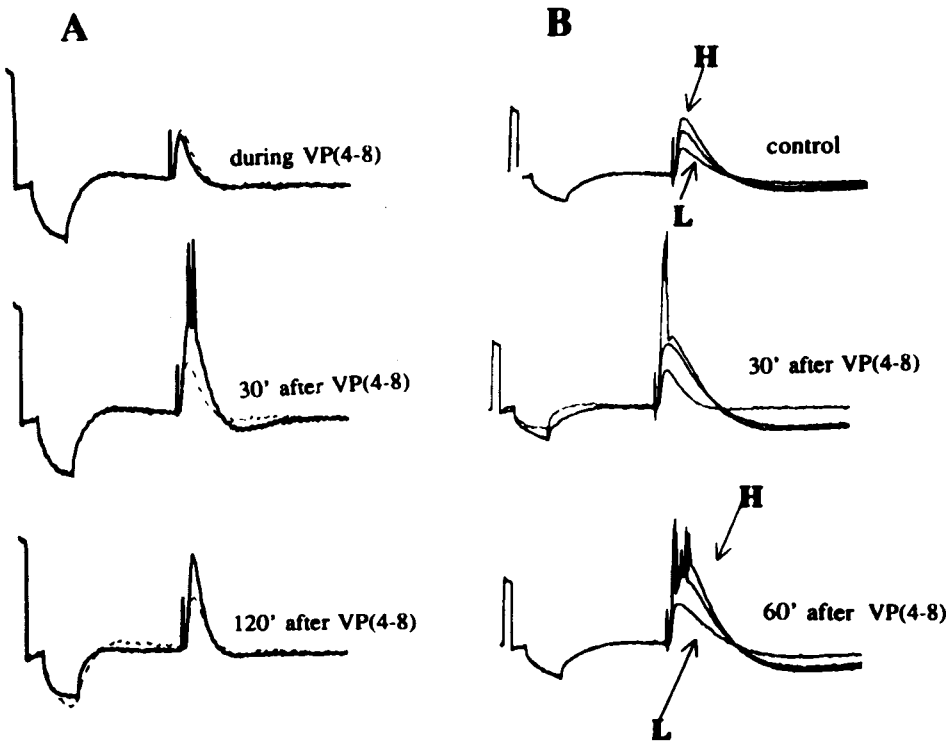

Fig. 2. (A) Example of the effect of 0.1 nM VP 4–8 on average ($n = 10$) EPSPs in one neuron of the CA_1/subiculum in the ventral hippocampus before, during (15 min), 30 and 60 min after the start of the 20-min peptide administration. Note the appearance of action potentials at 15 min during the administration of VP 4–8, truncated by averaging. The response to a hyperpolarizing current of 0.12 nA in the neuron was not markedly changed during the entire recording episode. The resting membrane potential in this neuron was −68 mV. (B) Example of the effects of 0.1 nM VP 4–8 on average EPSPs ($n = 10$) in another neuron, studied in the presence of 50 μM picrotoxin and D-APV in the medium, during a 20 min bath application of 0.1 nM VP 4–8, at 30 and 60 min after the start of peptide administration. Superimposed are responses to three different stimulus intensities: high (H), medium (middle sweep in the all panels), and low (L). At 30 and 60 min, the high and medium stimulus intensities (the middle sweep in lower panel) produced action potentials above the EPSPs while the EPSPs elicited by weak stimuli remained under the firing threshold of the neuron. The action potentials in response to the high and medium stimulation were truncated by averaging. The resting input resistance of the neuron was not markedly altered during the experiment, as indicated by the responses to a hyperpolarizing current of 0.15 nA. The resting membrane potential of this neuron was −68 mV. Calibration: 15 mV, 5 ms (A); 10 mV, 5 ms (B) (reproduced with permission from Chepkova et al., 1995).

The failure of the V_{1a} receptor antagonist, $d(CH_2)_5$-Tyr-(Me)-arginine^8-VP, to block the VP-induced increase in EPSPs of lateral septum neurons (Van den Hooff et al., 1990a; Van den Hooff and Urban, 1990b) surprised us as the direct, excitatory membrane effects of the peptide on these neurons (Raggenbass et al., 1987a; Van den Hooff et al., 1990a) and on other neurons (e.g., Ma and Dun, 1985; Peters and Kreulen, 1985; see Kolaj and Renaud, this volume) were prevented by this antagonist. In addition, the V_{1a} receptor antagonist appeared to block several other central effects of VP, including the effect on social recognition (see Dantzer, this volume; Landgraf et al., this volume), but the effect of VP and related peptides on avoidance behavior can be blocked by a V_1 as well as an OT receptor antagonist (De Wied, 1991), flanking behavior (see Albers and Bamshad, this volume), pair bonding (see Wang et al., this volume), as well as the intracellular responses to VP in cellular

systems (see Thibonnier et al., this volume; Jurzak and Schmid, this volume), which were elicited by a broad scale of different doses of the peptide. In addition, $d(CH_2)_5$-Tyr-(Me)-arginine^8-VP blocked the VP-induced long-lasting potentiation of field EPSPs in the CA1 area of the hippocampus (Chen et al., 1993), an effect that closely resembled the peptide-induced facilitation of EPSPs in our intracellular experiments. Moreover, the same antagonist suppressed the responses to glutamate (Urban and Killian, 1990) and acted as general depressant in spinal cord neurons (Porter and Brody, 1986), suggesting that the blockade of some of the VP effects on the brain neurons might have been caused by an unspecific, depressant action of this antagonist. The fact that even a ten-fold higher concentration of this antagonist failed to prevent the facilitating effect of VP on EPSPs in lateral septum neurons, in spite of its unspecific depressant action, suggests that the batch of antagonist used might not have been effective. However, when tested in the bioassay performed, the same batch of antagonist prevented the VP-induced rise in blood pressure (unpublished observation), indicating that the peptide antagonist was indeed effective.

The potentiating effect of VP on EPSPs might develop after metabolism of VP into active metabolites, detectable in the hippocampus (Stark et al., 1989). Alternatively, it could be a result of an action of VP on binding sites specific for VP 4–8/4–9. However, no sites binding shorter VP metabolites has not yet been found in the hippocampus; although the first direct evidence for sites specifically binding [^{35}S]-VP 4–8 is presented in this volume (see Du et al., this volume). Autoradiography with [^{3}H] and/or [^{125}I] ligands for the vascular V_1 type of VP receptor and for OT receptor revealed that V_1 VP and OT receptors can be clearly distinguished in the hippocampus and other brain areas. The dentate gyrus of the hippocampus appears to contain predominantly high-affinity V_1 type VP receptors (Kremarik et al., 1993), while the subiculum/CA_1 field of the ventral hippocampus is rich in OT binding sites which have the same affinity for OT and VP (Elands et al., 1988; Phillips et al., 1988). Thus, OT receptors might be involved in the EPSP increase elicited by VP and VP 4–8. However, neither VP 4–8 nor VP 4–9, administered in a dose 10^3 higher than the effective dose of OT, produce OT-like effects on uterine muscle in vivo (De Jong et al., 1985). In addition, VP 4–9 fails to displace a V_1 receptor antagonist from its binding in the hippocampus (Phillips et al., 1988). The binding sites for VP 4–9 in the brain, as demonstrated with either [^{35}S]arginine^8-VP (Brinton et al., 1985; De Kloet et al., 1985) or monoclonal anti-idiotypic antibodies (Jurzak et al., 1993), differ from the sites for VP/OT. These results and the recent finding of specific binding sites for VP 4–8 in synaptosomal membrane preparations from the anterior cortex (Du et al., 1994; for review, see Du et al., this volume) suggest strongly that many of the central effects of VP and its metabolites are mediated by specific receptor-complex(es) distinct from the peripheral V_{1a}, V_2 or OT receptors. Further studies with various agonist ligands for VP and for OT receptors might prove helpful for the characterization of the receptors mediating the effect on EPSPs.

The intracellular effector pathway mediating the VP- and VP 4–8-induced long-lasting enhancement of the EPSPs evoked in the CA/subiculum cells by stimulation of the stratum radiatum remains to be elucidated. The increase in the slope and the amplitude of these glutamate-mediated EPSPs (Collingridge et al., 1988) was not associated with a change in the RMP and/or $R_{(IN)}$ of the neurons, suggesting that the peptides, in the concentration used, did not activate receptor-gated conductances. A typical feature of the NMDA receptor-mediated long-term potentiation (LTP) of these EPSPs in the CA_1 of the hippocampus is that they depend on an influx of Ca^{2+} through either the NMDA receptor-channel complex and/or voltage-gated calcium channels. This Ca^{2+} influx is thought to initiate intracellular processes that lead to an increase in glutamate release, and to enhance the sensitivity of postsynaptic glutamate receptor-channel complex (for review, see Bliss and Collingridge, 1993). VP and VP 4–8 still facilitated EPSPs in the presence of $GABA_A$ and NMDA receptor antagonists. Decreased synaptic inhibition or increased Ca^{2+} influx through NMDA receptors is therefore unlikely to be the mechanism for the EPSP-potentiating action of the peptides. However, VP and VP 4–8 stimulate inositol phospholipid

metabolism in neurons (Shewey and Dorsa, 1988; Moratalla et al., 1989), mobilize Ca^{2+} from internal stores in cultured hippocampus neurons and in vascular smooth muscle cells (Van Renterghem et al., 1988; Brinton et al., 1994; see also Du et al., this volume), and increase transmitter release (Abdul-Ghani et al., 1990; Maegawa et al., 1992). Thus, VP and VP 4–8 might have facilitated the EPSPs through a Ca^{2+}-dependent mechanism, leading to an increase in glutamate release. It should, however, be noted that a Ca^{2+}-induced increase in the sensitivity of postsynaptic receptors for glutamate, similarly to that shown by LTP (Bliss and Collingridge, 1993) could account equally well for this effect of the peptides.

In most of the neurons studied, the peptide-induced increase in EPSPs was associated with a shortening of the rising phase of the voltage response to depolarizing pulses, a decrease in the threshold and an increase in the number of action potentials elicited by sustained depolarization, suggesting that the conductances controlling the membrane response to a voltage step were affected by 0.1 nM VP peptides. The delay in firing the first action potential, the threshold, and number of action potentials in hippocampal neurons in response to depolarizing current injection are predominantly determined by various components of the outward potassium current, such as the rapidly inactivating I_A, the rapidly activating and slowly inactivating I_D, and the Ca^{2+}-dependent I_{AHP} (Gustafsson and Wigström, 1981; Madison and Nicoll, 1984; Segal et al., 1984; Lancaster and Adams, 1986; Storm, 1988, 1989; Alger, 1990). Partial suppression of this current by VP or VP 4–8 in postsynaptic neurons would presumably decrease the delay and threshold and increase the number of action potentials during depolarization. Suppression of the outward potassium current in postsynaptic neurons might also be expected to decrease the threshold and increase the number of action potentials triggered by EPSPs, an effect that was seen in several of the neurons thus tested. The same effect in presynaptic terminals would, presumably, increase the probability of transmitter release as a result of increased probability of invasion of the terminals by action potentials. VP closes ATP-sensitive K^+-channels in cultured smooth muscle cells (Wakatsuki et al., 1992) and in insulin secreting cells (Martin et al., 1989). This and other effects of VP on brain neurons, such as reversible depolarization, increase in input resistance and spontaneous activity, and activation of a voltage-dependent inward current (Mizuno et al., 1984; Ma and Dun, 1985; Peters and Kreulen, 1985; Widmer et al., 1992; Raggenbass et al., 1993), could thus be a result of different intracellular effector pathways being activated by these peptides via the same or different membrane receptors. Activation of three different intracellular effector pathways by activation of the same VP receptor has been demonstrated (Van Renterghem et al., 1988; Kremer et al., 1992). The effective concentration of the peptide may be one of the ways of distinguishing between these effects. Voltage-clamp analysis in combination with intracellular manipulation of diverse messenger systems is needed to obtain a better understanding of the role of the effects of VP and related peptides on the functioning of the brain neurons.

Synaptic responses in lateral septum increased by medial forebrain bundle stimulation

Electrophysiological experiments attempting to study the function of VP in vivo were rare. Only the experiments of Smock and coworkers (see Smock et al., this volume) have addressed this issue. The VP- containing fibers in the hippocampus originate, among others, in the medial nucleus of the amygdala (Caffé et al., 1987). Earlier studies with brain slices, showed that 1 μM VP in the bath depressed the population spike evoked in the CA1 field of the hippocampus by stimulation of the stratum radiatum (Albeck and Smock, 1988), presumably by activating the inhibitory interneurons (Mühlethaler et al., 1984). Brief trains of 100 Hz stimulation to the medial amygdala nucleus depressed the population spike in the CA1 field of the hippocampus the same way as does VP. Thus induced population spike suppression lasted only a few seconds to less than a minute after the stimulation, and decreased on repeated stimulation (Albeck et al., 1990), suggesting that this depression was a result of short-lasting excitation of inhibitory interneurons. However, the amygdaloid stimulation effect was not blocked by a V_1 receptor antagonist

that blocked the depressant action of VP (Smock et al., 1990). Interestingly, fEPSPs and other postsynaptic potentials in the CA1 field were not affected by the amygdala stimulation (Smock et al., 1991).

The vasopressinergic fibers, which terminate in the lateral septum complex, originate from the bed nucleus of the stria terminals (BNST) and from medial amygdaloid nucleus (Buijs, 1978; Sofroniew, 1985; De Vries and Buijs, 1983; Fliers et al., 1986; Caffé et al., 1987). They enter the complex ventrally through the medial forebrain bundle and diagonal band of Broca (DBB) that also conveys noradrenergic, dopaminergic and serotonergic fibers to the septum complex (Lindvall and Bjorklund, 1974; Lindvall and Stenevi, 1978; Kohler et al., 1982). A number of findings (Buijs and Swaab, 1979; Buijs and Heerikhuize, 1982; Land et al., 1982; Lu et al., 1982; Segers and Burbach, 1987a,b; see also Landgraf et al., this volume) have suggested that VP and other products of the prohormone, propressophysin, such as neurophysin II and C-terminal glycopeptide (CPP1-39), are released synaptically in the lateral septum. We examined in experiments on urethane-anesthetized rats, whether electrical stimulation of the ventral fiber input to the lateral septum complex, which conveys VP fibers to the lateral septum, is able to alter the synaptic excitability of the lateral septum neurons in the same way as does VP. We used male Brattleboro rats that were either heterozygous (HE) or homozygous (HO) for diabetes insipidus (DI), and males of the Long–Evans strain. Electrical stimulation of the afferent fibers in the fimbria bundle elicits in neurons of the dorsolateral septum, an EPSPs-IPSPs sequence that extracellularly generates negative (N)-positive (P) field potentials (FPs). The leading N-wave of these FPs reflect the EPSPs and action potentials current whereas the P-wave is generated by IPSPs current (De France et al., 1973a,b). We used thus-induced FPs to study the effect of DBB and BNST stimulation on the excitability of the lateral septum neurons.

A series of 15 stimuli was administered at a frequency of 1/min to the fimbria fibers. The FPs evoked were digitized and stored in a personal computer. Using an appropriate program we computed, printed and stored: (1) the maximum amplitude of the N- and P-waves, measured from the 0 V level; (2) the latency between the N- and P-wave maxima and the stimulus artifact. Also computed and plotted were the average FPs of each of the stimulation series. The mean (±SEM) amplitude of the N- and P-waves in each of the stimulation series were printed. The amplitudes of the N- and P-waves in the average FPs obtained in each of the stimulation series were expressed as percentages of the amplitude of the FPs waves in the first series of stimuli. Four to five series of FPs were recorded prior to DBB stimulations to ascertain the stability of the response. Immediately after the last control stimulation (time $t = 0$ min), the DBB or BNST was stimulated for 3 min with a stimulation pattern consisting of 30 s trains of square-wave pulses of 8 Hz frequency and 250 μA current intensity, alternating with 30 s periods of rest. This stimulation pattern resembles the phasic firing patter of VP-producing neurosecretory neurons (Poulain and Wakerley, 1982; Andrew, 1986), however it is ineffective to induce either LTP or long-term depression (LTD) forms of long-lasting plasticity in glutamatergic pathways in the hippocampus (Dudek and Bear, 1992). The recording of FPs was continued for 180 min following stimulation. At the end of the experiment, a (+)DC current of 100 μA was passed for 15 s through the stimulating and recording electrodes. This current deposited sufficient iron in the tissue to allow the subsequent histological localization of the electrodes with a prussian-blue reaction.

Daily water consumption and urine production were significantly higher in HO-DI rats than in HE-DI litter-mates and Long–Evans rats from which the Brattleboro rats descend (Valtin, 1967) (see Table 1). This demonstrates the expected severe water retention deficiency in the HO-DI rats, that lacks the pituitary and brain VP.

The amplitudes of the N waves in FPs decreased by 5–10% in the course of 4-h recordings in a group of control Long–Evans rats (Fig. 3). These rats had the stimulating electrodes inserted in the DBB but the DBB itself had not been stimulated. The amplitude of the P wave decreased by 10–20% in the non-stimulated rats. In total, 15 Long–Evans male rats were stimulated in the DBB. In two of these rats, the FPs after DBB stimulation showed only a time-dependent decrease comparable to that in the

Table 1
Mean (±SEM) water consumption and urine production (in ml/24 h) in Brattleboro rats homozygous (HO) and heterozygous (HE) for diabetes insipidus (DI) and in Long–Evans (LE) rats

Rat	No. of animals (n)	Water	Urine
HO-DI	12	150.6 ± 5.9[a]	117.5 ± 5.6[a]
HE-DI	11	48.6 ± 4.2	9.5 ± 0.6
LE	11	34.4 ±1.3[b]	10.5 ± 0.9

[a] Significantly different ($P < 0.001$ in Student's t-test) from HEDI.
[b] Significantly different ($P < 0.005$) from HE-DI.

control, non-stimulated rats. The stimulating electrodes appeared to be located in the part of the DBB rostral to the septum. These rats were therefore not included in the final analysis. The stimulation sites in the remaining 13 rats were, at least partly, in the ascending column of the middle or the posterior part of the DBB, 2.0–3.5 mm above the base of the brain and 0.5–1.5 mm from the midline. In 9 of these rats, the N wave of the FPs increased by 5–42% as a result of the DBB stimulation. However, in two of the remaining rats, the N-wave showed no noticeable change after the stimulation, and in two other rats, the N-wave decreased with the result that the amplitude of the N-wave of these rats at the end of the experiment was 10–15% below that of the controls. Although this lowered markedly the mean increase of the N-wave in the stimulated rats in a MANOVA test for repeated measurements, the mean amplitude of the N-waves in stimulated rats ($n = 13$) still differed significantly from that of non-stimulated rats (see Fig. 3). The effect was mostly evident in the first FPs series recorded 15 min after stimulation. In a group of 11 rats that showed the increase, the effect became significant ($P < 0.05$ in post-hoc Wilcoxon matched-pair signed rank test) at 30 min, reached its maximum at 45–60 min ($P < 0.02$), often staying at this level for the rest of the experiment (i.e., over 3 h). At the end of the experiment, the N-wave in these rats was 5–43% above the average N-wave of the non-stimulated rats (see Fig. 3). In most instances the P-waves changed in accordance with the N-wave though the change in these waves was generally smaller and very variable (Fig. 3).

The subsequent 5 experiments provided an explanation of the stimulation-induced decrease in FPs, observed in 2 rats of the first series of experiments. In these experiments FPs were recorded simultaneously from two lateral septum sites and it was found that the FPs derived from one of the electrodes increased after the stimulation while those obtained from a second electrode either increased ($n = 1$), showed no change ($n = 2$), or decreased ($n = 2$). Fig. 4 depicts one of these experiments in which the opposite effect on FPs of the DBB stimulation was found. Note that the change in both FPs recorded 390 min after DBB stimulation was almost equal to the change seen 60 min after stimulation. Thus, the stimulation effect may remain limited to a discrete lateral septum area that could easily be missed with a single electrode recording, as was used in most of the experiments.

To elicit an effect on FPs in the lateral septum, the site of stimulation in the DBB appeared to be critical, as is shown by the experiment summarized in Fig. 5. Of two stimulations in this experiment, only that in a dorsolateral segment of the DBB increased the N-wave in the FPs in the lateral septum. The N-wave in this rat showed a marked decrease immediately after DBB stimulation. However, in the subsequent 15 min the N-wave increased just as in other rats. A similar, short-lasting N-wave decrease following DBB stimulation was also observed in 5 other rats. Stimulation of the fimbria fibers for 3 min with the intermittent 8 Hz stimulus pattern had no effect on the FPs and elicited no LTP-like N-wave increase. However, stimulation of the fimbria fibers with 50 Hz for 5 s resulted in a further, LTP-like increase of the N-wave in the FPs (Fig. 5). The N-wave increase elicited with the 50 Hz stimulation reached its maximum immediately after the stimulation and was sustained until the end of the experiment. In this and in three other experiments, the fimbria fibers caudal to the stimulating electrodes were cut before the experiment in order to demonstrate that connections of the septum with the hippocampus were not essential for the effect of DBB stimulation on the FPs.

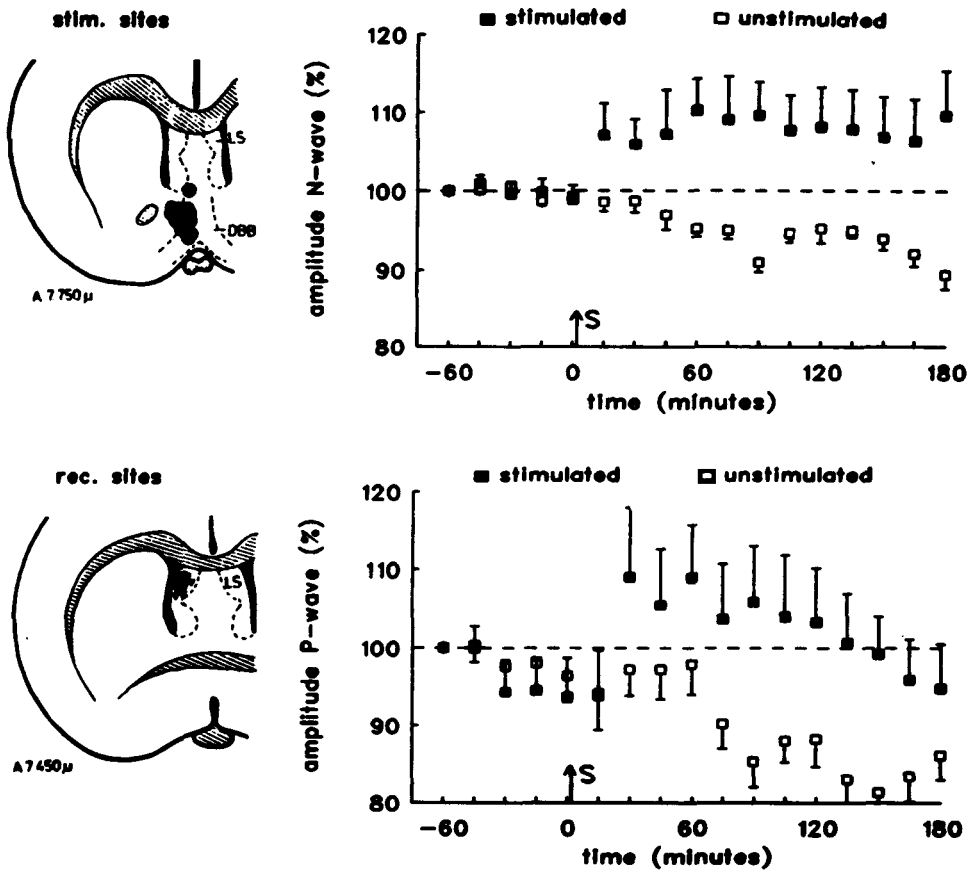

Fig. 3. The mean percent change (±SEM) in amplitude of the N- and P-waves in average field potentials (FPs), evoked in the LS by stimulation of the fimbria fibers, that occurred in the diagonal band of Broca (DBB) in non-stimulated, control male Long–Evans rats ($n = 7$) and in 13 stimulated Long–Evans rats. The stimulation (S) was applied at time 0 min. For example of the FPs see Fig. 4. The stimulation (STIM) sites in the DBB and the recording (REC) sites in the lateral septum (LS) are illustrated on frontal sections drawn on the left (source: J.R.F. Koning and R.A. Klippel, *Stereotaxic Atlas*, Williams and Wilkins, New York, 1963). MANOVA comparison showed a significant difference between stimulated and non-stimulated groups (main effect $F(20;1) = 5{,}72$; $P = 0.027$). There was neither a time effect ($F(220;11) = 0.57$; $P = 0.853$) nor treatment × time effect ($F(220;11) = 1.46$; $P = 0.148$). In a group of 11 rats that showed the increase, it became significant at 30 min ($P < 0.05$ in post-hoc Wilcoxon matched-paired signed rank test), reached its maximum at 4560 min ($P < 0.05$) often staying at this level for the rest of the experiment (i.e., over 3 h).

The results of DBB stimulation in HE-DI rats were similar to those obtained in Long–Evans rats. Five of seven HE-DI rats showed a 5–31% increase in the N-wave 60 min after the stimulation. The remaining two rats showed no or only a small increase in the FPs after the stimulation. However, none of the 9 HO-DI rats stimulated in the DBB showed a change in the FPs in the lateral septum following stimulation (Fig. 6). The MANOVA test for repeated measurements showed a significant difference between the means of the N-wave in HE-DI and HO-DI rats after stimulation.

We stimulated BNST in fifteen Long–Evans rats, with the same stimulus pattern as used in the DBB stimulation experiments, and recorded FPs in lateral septum. The results are summarized in Fig.

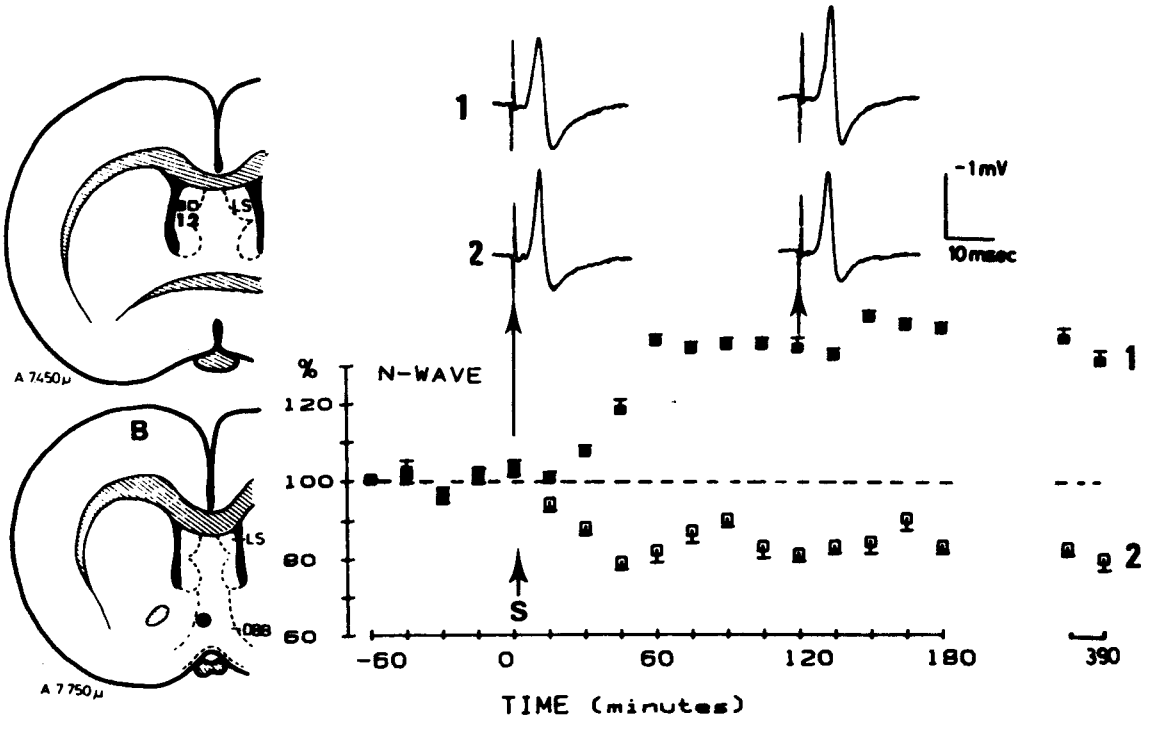

Fig. 4. Example of opposite effects of DBB stimulation on FPs in two different sites in the LS of Long–Evans rats. Plot shows the mean (±SEM) percent amplitude of the N wave in the FPs that were recorded with electrode 1 (filled symbols) and electrode 2 (open symbols) from the sites in the lateral septum (LS) as indicated in the upper left insert. The site of stimulation (S) in the DBB is indicated with a filled symbol in the lower left insert. Inserts 1 and 2 represent the average FPs ($n = 15$) from electrodes 1 and 2 at the time indicated by arrows.

7. Ten of these animals showed a clear, long-lasting increase in the amplitude of the N-wave in the FPs after the stimulation, FPs did not change in 3 rats and decreased gradually in two other rats being approximately 10% below baseline at 180 min after stimulation.

The principal finding in the experiments presented here was that in most of the Long–Evans and HE-DI Brattleboro rats, an intermittent, 8-Hz stimulation in specific sites in the DBB and BNST potentiated for several hours the N-wave in the FPs that were evoked in the lateral septum by stimulation of the fimbria fibers. The N-wave is built up from EPSPs and action potential currents generated in lateral septum by the stimulation while the current of the IPSPs is considered to be the main source of the P-wave (DeFrance et al., 1973a,b). Thus, the increase in the N-wave indicates that stimulation at both sites increased the excitability of the lateral septum neurons in response to the glutamatergic synaptic input from the fimbria fibers.

The response of lateral septum neurons to stimulation of the fimbria fibers consists of EPSPs, with or without action potentials, followed by IPSP (Van den Hooff et al., 1989a). The close time relation between the EPSPs and IPSPs and the block of the latter potentials by picrotoxin or bicuculline (Van den Hooff et al., 1989a) supports the notion (Gutnick and Feldman, 1977) that the IPSPs result mainly from a GABA-ergic, presumably recurrent, inhibition. Indeed, bicuculline also almost completely blocked the P-wave in the FPs (Joëls and Urban, 1984c). Thus, no other currents than the $GABA_A$ receptor-induced membrane currents in the lateral septum cells, such as the hyperpolarizing

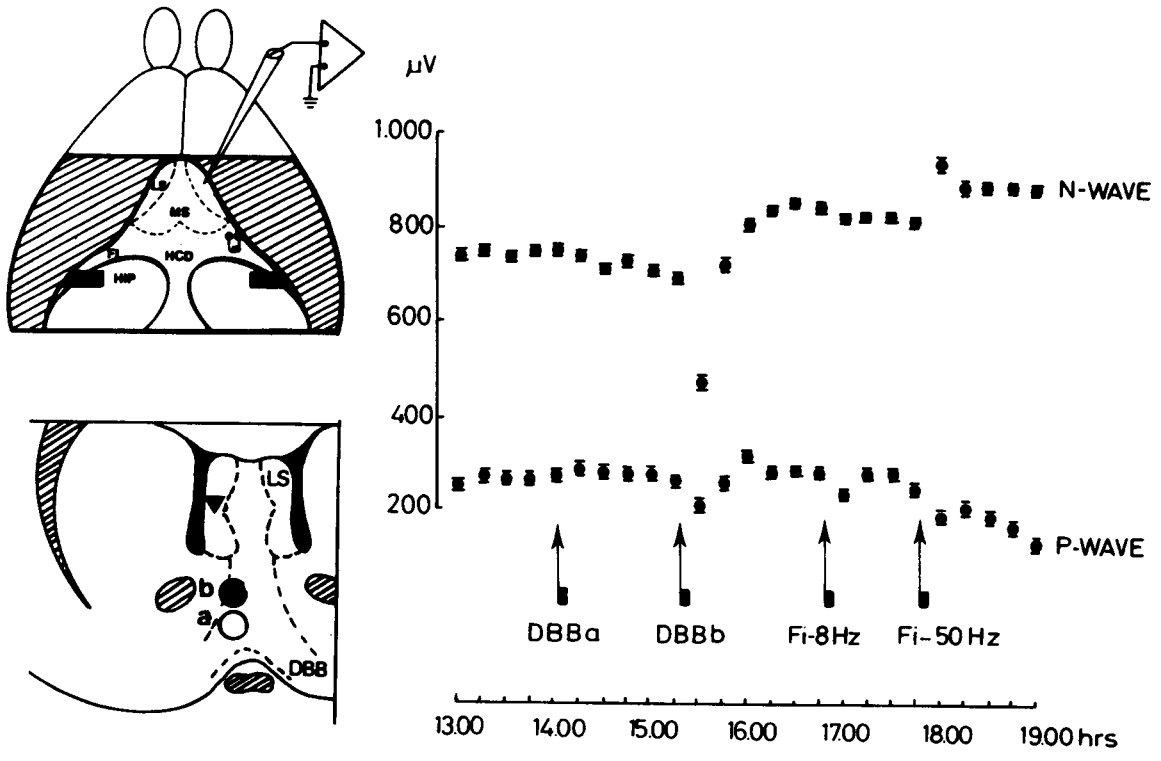

Fig. 5. Example of the effect of diagonal band of Broca (DBB) stimulation on FPs in the LS in a Long–Evans rat in which the fimbria fibers had been lesioned at a site indicated by bars in the left insert. The shaded area in the upper insert indicates the extent to which the neocortex and the corpus callosum had been removed. Stimulation sites a and b are depicted in the lower insert, the triangle symbol indicates the recording site in the LS. Plot shows the mean amplitude (±SEM) of the negative (N) and positive (P) waves before and after stimulation in the DDB at site a (arrow DDBa) and site b (arrow DBBb). At the time indicated by Fi8 Hz, the fimbria fibers were stimulated for 3 min according to the intermittent 8-Hz stimulation setup. The Fi50 Hz arrow indicates 5-s, 50-Hz stimulation of the fimbria fibers. HIP, hippocampus; MS, medial septum.

afterpotential or bicuculline sensitive-slow IPSPs (for review, see Gallagher et al., 1995), seem to contribute to the P-wave. The P-wave, along with the N-wave, rose after the DBB stimulation. The stimulation-induced increase in the N-wave thus could not be due to a decrease in the recurrent inhibition, but was almost certainly due to an increase in the synaptic excitability of the lateral septum neurons induced by the fimbria fibers.

In 30–40% of the rats, DBB stimulation failed to affect the FPs. This was presumably due to the diffuse distribution of VP-ergic fibers in the ascending columns of the DBB (Buijs, 1978), making it difficult to activate a sufficient number of the fibers or neurons in each of the rats stimulated. Fig. 5 illustrates the importance of the stimulation site in the DBB for eliciting the effect on FPs. In addition, stimulation of a set of fibers in the DBB may release peptides only in a strictly localized small part of the lateral septum. In this part of the septum, only the N-wave, and thus synaptic excitability, will increase after stimulation. As a result of increased recurrent inhibition at the site of increased transmission, adjacent parts of the lateral septum, either will show no change in the N-wave, or the N-wave in those sites may even decrease after stimulation (see Fig. 3). Fibers in the medial segments of the fimbria bundle terminate in the dorso-medial areas of the lateral septum while those in more lateral segments terminate in ventrolateral lateral septum

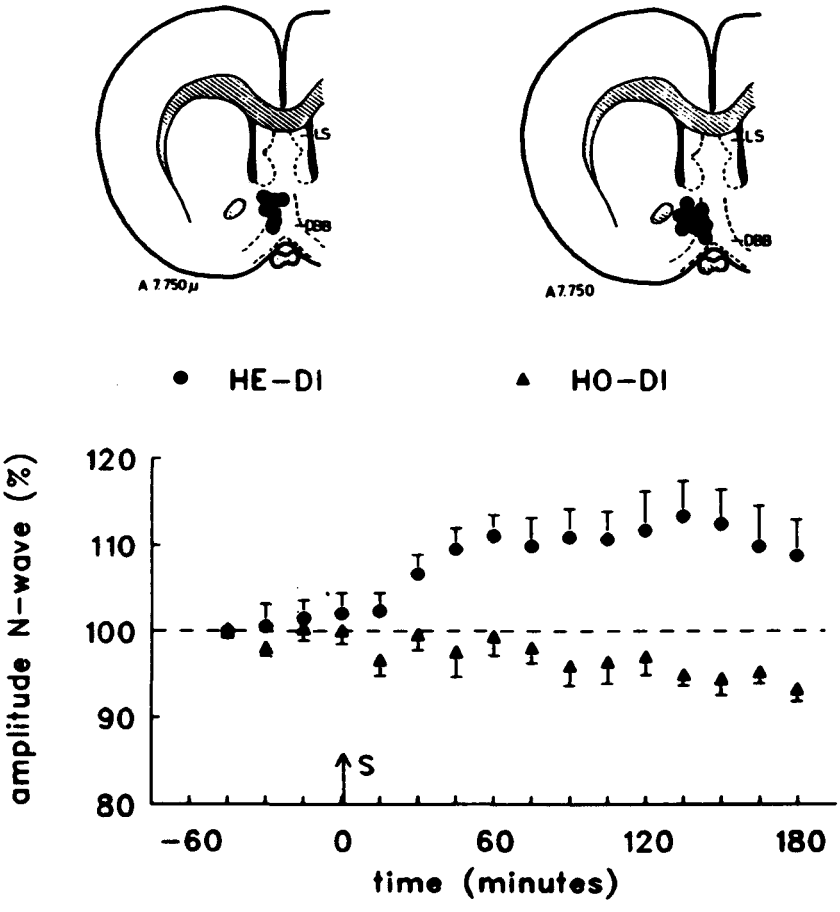

Fig. 6. The effect of stimulations (S) in the diagonal band of Broca (DBB) on the N wave in average FPs recorded from the LS of Brattleboro rats heterozygous (HE) ($n = 7$) and homozygous (HO) ($n = 9$) for DI. The stimulation sites are illustrated in the drawings from the source indicated in the legend to Fig. 1. The MANOVA for repeated measurements showed a significant difference (main effect $F(14;1) = 15.78$; $P < 0.01$) between the means of the N-wave in HE-DI and in HO-DI rats after stimulation.

areas (Meibach and Siegel, 1977; Swanson and Cowan, 1977). As a consequence, each set of the fimbria fibers may be expected to excite neurons and to evoke large N-wave FPs in different loci of the lateral septum. Outside the locus of synaptic excitation the P-waves of the FPs mostly were larger than the N-waves. It has been found that, with increasing intensity of stimulation, especially the P-wave increased in areas around the focus of excitation (Joëls and Urban, 1985a), indicating that synaptic inhibition has increased in those areas. Further evidence of strong, recurrent inhibition in the lateral septum was provided by intracellular recordings. Specific fimbria fibers had to be stimulated when large EPSPs were to be elicited in a lateral septum neuron. Stimulation of other fimbria fibers promoted IPSPs while the EPSPs decreased (De France et al., 1973a,b).

Long-lasting potentiation of transmission in excitatory synapses like those on the lateral septum neurons, for which excitatory amino acids are instrumental, can be elicited by brief 50 Hz stimulation of these synapses. This kind of transmission potentiation, designated as LTP by Bliss and Lomø

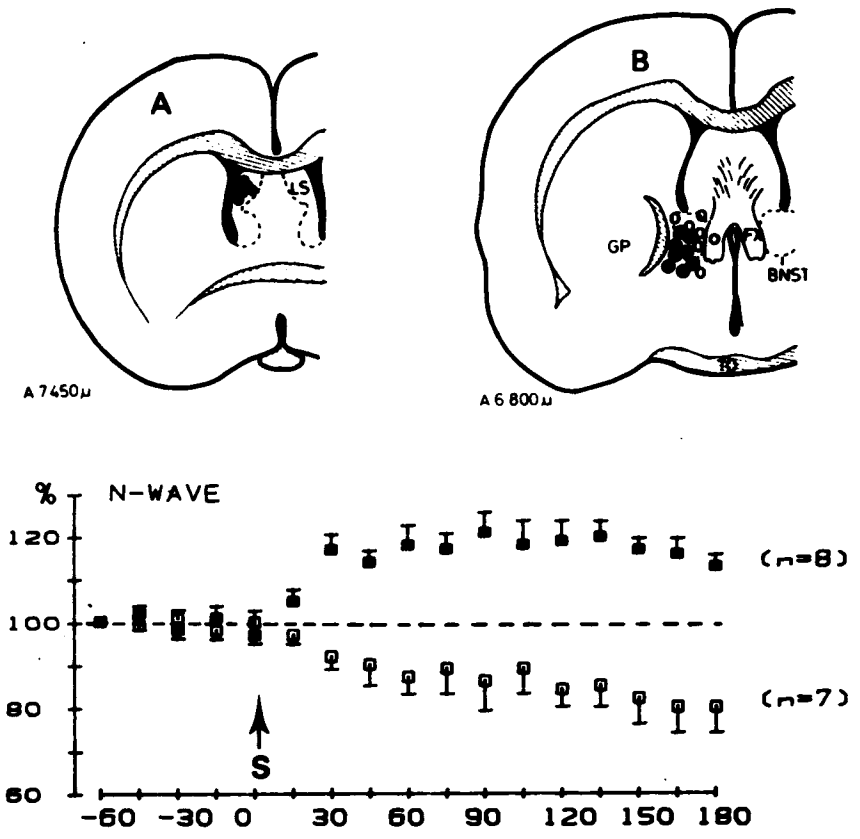

Fig. 7. (A) The mean percent change (±SEM) in amplitude of the N wave in the average FPs in the LS of Long–Evans rats (n = 9) following stimulation (S) in the Bed nucleus of the stria terminalis (BNST). The stimulation sites are illustrated in the left insert.

as LTP (Bliss and Lømo, 1973), is thought to be caused increased release of transmitter and increased sensitivity of the postsynaptic glutamate receptors (Bliss and Collingridge, 1993). A 50-Hz stimulation of the fimbria fibers for 2–5 s in these and in other experiments in vitro (Van den Hooff et al., 1989b), elicited a LTP-like increase in transmission of excitation from these fibers on lateral septum neurons. Stimulation of the fimbria fibers at frequencies below 10 Hz, both in the present material and in lateral septum slices (Van den Hooff et al., 1989b), appeared to be ineffective in this respect. Therefore, it seems evident that the increase in synaptic transmission between the fimbria fibers and lateral septum neurons elicited by DDB and BNST stimulation was mediated by a principle other than an excitatory amino acid.

Several findings suggest that this principle might be VP and/or other propressophysin peptides. The increase in time course, magnitude and duration of the stimulation-induced N-wave in FPs strongly resembled that elicited by VP (Urban and De Wied, 1986). The DBB and BNST sites, stimulation of which potentiated the synaptically evoked FPs in the lateral septum of Long–Evans, HE-DI Brattleboro and Wistar rats (unpublished observation), corresponds well with the pathway of the VP-containing or CPP-immunoreactive fibers projecting to the lateral septum (Buijs, 1978; Dubois-Dauphin and Zakarian, 1987). In HO-DI Brattle-

boro rats, which do not produce VP, the same DDB stimulation failed to affect the N-wave in FPs. The plots of the average changes in the N-wave amplitude in HO-DI Brattleboro rats strongly resembled the plots obtained in the non-stimulated Long–Evans control rats (see Fig. 3). Such a result would have been expected if the increase, or the decrease, in the FPs that we found in normal rats following DBB stimulation was indeed caused by VP released in the lateral septum by the stimulation. Exogenous VP, applied in a 10^{10} M concentration to the dorsal surface of the septum, increased the N waves in the FPs in the lateral septum in nearly the same way as did DBB stimulation (Urban and De Wied, 1986). In lateral septum slices, as little as 10^{-12} M to 10^{-10}VP or CPP for a long time potentiated the EPSPs evoked in lateral septum neurons by stimulation of the fimbria fibers (Van den Hooff and Urban, 1990a,b). The total, calcium-dependent release of VP from the lateral septum, elicited in conscious rats by osmotic or hypovolemic stimuli, amounted to 80–100 pg of the immunoreactive peptide (Demotes-Mainard et al., 1986). The VP release from the lateral septum of rabbits was of similar magnitude (Disturnal et al., 1984). Thus, a 10^{-11} to 10^{-12} M concentration of VP locally in the lateral septum seems to be quite physiological.

General conclusions

There is growing evidence that minute concentrations of VP and metabolites released from propresophysins can enhance for hours the excitability of lateral septum and hippocampus neurons in response to a synaptic, glutamate-mediated input. Nootropic drugs, that potentiate the non-NMDA receptor-mediated glutamatergic transmission in the hippocampus, markedly improve performance of odor discrimination and water-maze tasks (Staubli et al., 1994), suggesting that potentiation of transmission via these receptors may be important in the role played by the hippocampus in many forms of learning and memory. Thus, the memory enhancing effect of VP and its metabolites could be due to the enhancing effect of these peptides on non-NMDA glutamate receptor-mediated EPSPs. However, the block of the peptide effect with antagonists for the non-NMDA glutamate receptor needs to be demonstrated to support this notion. This, presumably, physiologically significant action of the peptides in brain (Urban, 1987b) may be complementary to LTP, increasing transmission plasticity in those brain structures that are innervated by VP-releasing fibers. The facilitation by VP of LTP expression in the lateral septum (Van den Hooff et al., 1989b) and the electron microscopy finding of VP-containing synapses in close vicinity of glutamate containing terminals of the fimbria fiber on dendrites of the lateral septum neurons (Jakab et al., 1991) support this notion.

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CHAPTER 3.2.4

Electrophysiological studies of neurohypophysial neurons and peptides

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We have used hypothalamic slices of the supraoptic nucleus (SON) to investigate synaptic control of magnocellular vasopressinergic and oxytocinergic neurons. With the use of perforated patch recording techniques we identified and isolated excitatory or inhibitory postsynaptic currents elicited by electrical stimulation of afferent fibers. Both inhibitory and excitatory afferent fibers displayed presynaptic $GABA_B$ receptors; the $GABA_B$ agonist, baclofen caused a dose-dependent suppression of the evoked potentials in the absence of any effects on postsynaptic input resistance. Further evidence for a presynaptic locus included an increase in paired pulse ratio and a lack of effect on currents elicited by exogenously applied muscimol (a $GABA_A$ receptor agonist) or AMPA (a glutamate agonist). With the use of an $GABA_B$ receptor antagonist we demonstrated an action of endogenously released GABA, acting at $GABA_B$ receptors on excitatory terminals, to reduce excitatory transmission. In addition to presynaptic modulation by GABA of afferent inputs, we also observed actions of vasopressin and oxytocin, released from dendrites of magnocellular SON neurons, to gate afferent, excitatory transmission in the SON. Exogenously applied vasopressin and oxytocin, or these peptides when released by depolarizing stimuli of magnocellular neurons, reduced the size of evoked excitatory postsynaptic potentials at a presynaptic locus. We have also observed actions of arginine vasopressin to modulate the action of glutamate in slices of the ventral septal area and to attenuate a glutamate-mediated excitatory postsynaptic current in slices of the parabrachial nucleus.

Introduction

The electrophysiology and pharmacology of hypothalamic magnocellular neurons has been studied extensively. Some 40 years ago, reports appeared indicating that the hypothalamic-neurohypophysial tract was capable of sustaining action potentials, suggesting that these neuroendocrine cells displayed significant 'neural' features (reviewed in Hayward, 1977). Shortly thereafter, Kandel reported on intracellular studies which revealed overshooting action potentials in goldfish magnocellular preoptic neurons (Kandel, 1964). With the advent of techniques for recording in slices and cultures, electrophysiological studies on mammalian supraoptic and paraventricular neurons have multiplied and membrane currents, afferent influences and functional properties have been well described (reviewed in Renaud and Bourque, 1991). One aim of this communication is to build on this extensive literature by reporting on our recent electrophysiological studies in the supraoptic nucleus (SON) concerning the action of the inhibitory amino acid neurotransmitter, GABA. In addition we will describe some interesting actions of the neuropeptides, oxytocin (OT) and arginine vasopressin (VP), both present in this nucleus.

In addition to their presence in the magnocellular SON and paraventricular (PVN) neurons and in their projections to the pituitary, VP and OT are also found in many parts of the central nervous

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system. This innervation arises from both extrahypothalamic VP-containing cell groups (i.e. in the bed nucleus of the stria terminalis) and from vasopressinergic and oxytocinergic parvocellular neurons in the PVN. Functional correlates of these innervations appear as actions on a wide variety of autonomic and behavioral states. Some of the better understood actions of VP within the brain include effects on behavior (De Wied et al., 1993; Engelmann et al., 1996), blood pressure (Pittman and Landgraf, 1991; Pittman and Bagdan, 1992) and temperature regulation (Zeisberger, 1985; Pittman and Landgraf, 1991). OT appears to have neural actions on reproductive (Richard et al., 1991) and social (Insel and Hulihan, 1995) behaviors and possibly on cardiovascular and osmotic control mechanisms as well (Morris et al., 1995; Stricker and Verbalis, 1996). The underlying mechanisms for these effects reside in the action of these peptides on neurons and neural circuits involved in these various functions. We will therefore also review some of our electrophysiological studies on the actions of VP on neurons in extrahypothalamic areas.

Electrophysiological studies in the SON

In order to record from magnocellular supraoptic neurons, we have utilized a transverse slice of the rat hypothalamus which is submerged and perfused constantly with an oxygenated, pH-balanced artificial cerebrospinal fluid. In our hands, such slices maintain viability for up to 2 days. We carry out intracellular current and voltage recordings using patch electrodes in the perforated-patch configuration. Rather than using the conventional, whole cell mode of recording, in which there is a high probability that the integrity of the intracellular constituents will be altered due to diffusion of pipette contents into the cell, we utilize the nystatin-patch technique, in which membrane integrity is maintained, but electrical access is accomplished via small pores perforated in the membrane under a tight electrical seal by the antibiotic, nystatin (reviewed in Akaike and Harata, 1994). Magnocellular neurons can be identified by their high spike thresholds, and the presence of a slow, time-dependent rise in potential to the first spike, little frequency adaptation and slow return to resting potential after termination of a depolarizing intracellular current pulse. These characteristics facilitated easy differentiation from interneurons, but we were unable to differentiate between VP and OT neurons. Previous investigators (Stern and Armstrong, 1995) working in slices from juvenile female rats have suggested that the presence of a rectifying potential elicited by hyperpolarizing pulses from depolarized potentials is indicative of an OT neuron; in our hands, working with mature male rats (200–220 g), few neurons display this feature. As approximately 50% of the SON neurons would be expected to be vasopressinergic in nature, it would appear unlikely that this feature is useful for identifying the peptidergic nature of magnocellular cells, at least in male rats.

Stimuli which activate the VP and OT neurons of the SON have been well described (Renaud and Bourque, 1991; Leng et al., 1992). Although there is little doubt that these neurons are capable of responding directly to local conditions in the extracellular space (e.g. local osmolarity; Oliet and Bourque, 1993), anatomical (Swanson and Sawchenko, 1983; Tribollet et al., 1985) and functional (Poulain and Wakerley, 1982; Cunningham and Sawchenko, 1991) experiments indicate that these cells also receive a rich afferent innervation. In particular, SON neurons appear to be strongly innervated by fibers containing amino acid neurotransmitters. Immunocytochemical and binding studies reveal the presence of receptors, fibers and terminals immunoreactive for either GABA or glutamate within the SON (Theodosis et al., 1986; Decavel and Van den Pol, 1990; Van den Pol and Trombley, 1993). Electrophysiological studies, both in vivo and in vitro, have confirmed physiological roles for these two transmitters. Both spontaneous and evoked postsynaptic potentials of an excitatory nature can be blocked with glutamate antagonists (Gribkoff and Dudek, 1990; Gribkoff, 1991; Hu and Bourque, 1991; Wuarin and Dudek, 1993) and similar inhibitory postsynaptic potentials can be blocked with a $GABA_A$ antagonist (Jhamandas and Renaud, 1986; Randle et al., 1986; Wuarin and Dudek, 1993). In our hands, when we stimulate an area adjacent to the medial-dorsal aspect of the SON in slices, all evoked postsynaptic currents

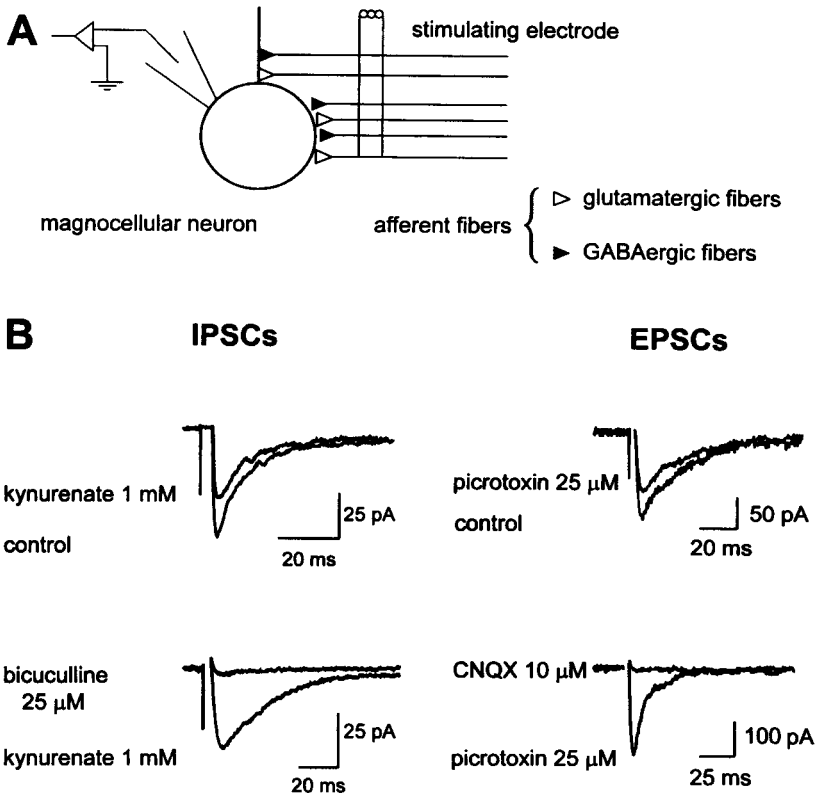

Fig. 1. (A) Schematic illustrating recording configuration, with a perforated patch type recording upon a magnocellular neuron in a coronal slice containing the SON. A nearby stimulating electrode placed adjacent to the SON is used to activate glutamatergic and GABAergic fibers innervating these neurons. (B) Isolation and identification of evoked currents in a cell voltage-clamped at −80 mV. On the left, an inward current is evoked which follows at short latency after the stimulation artefact (vertical transient); in the presence of the non-specific glutamate receptor antagonist, kynurenate, a pure GABAergic IPSC remains, as illustrated by its total block in the additional presence of the GABA antagonist, bicuculline. On the right, an inward current is evoked which is partially blocked by the GABA chloride channel blocker, picrotoxin. Remaining current is identified as an EPSC resulting from activation of AMPA type receptors, as revealed by its total block by the AMPA/kainate antagonist, CNQX.

appear to be mediated by activation of both glutamate and $GABA_A$ receptors (Fig. 1). Thus, perfusion with a non-specific excitatory amino acid antagonist, such as kynurenic acid, permits isolation of a pure, $GABA_A$-mediated inhibitory postsynaptic potential (IPSP) or current (IPSC). Conversely, by adding the $GABA_A$ chloride channel blocker, picrotoxin, or the GABA receptor antagonist, bicuculline, to the perfusate, we are able to isolate a pure, glutamate-mediated excitatory postsynaptic potential (EPSP) or current (EPSC).

Because it is now well accepted that neurons make extensive use of presynaptic receptors to influence circuit behavior and regulate neurotransmitter release, we became interested in the possibility that both glutamatergic and GABAergic afferent fibers innervating the magnocellular neurons could be under the control of presynaptically located GABA receptors. In particular, we investigated the role of the $GABA_B$ receptor (reviewed in Misgeld et al., 1995) in regulating excitability of afferent fibers innervating the magnocellular neurons of the SON (Kombian et

al., 1996). We found that baclofen, a $GABA_B$ receptor agonist, decreased GABA-mediated IPSCs with an EC_{50} in the nanomolar range (Fig. 2A). Similarly, fast glutamate-mediated EPSCs were attenuated by perfusion of baclofen at about the same concentration. As such effects could be mediated by actions either at pre- or postsynaptic sites, we also examined possible postsynaptic actions of baclofen on the magnocellular neurons. One possibility is that baclofen could interfere with the postsynaptic actions of the amino acid transmitters. This possibility was eliminated by the observation that postsynaptic membrane currents elicited by transient application of either a $GABA_A$ agonist, muscimol, or a glutamate agonist, AMPA, were unaltered by simultaneous application of baclofen (Fig. 2B). A second possibility was that baclofen altered other postsynaptic currents in such a way as to make less effective the synaptic inputs. However, at normal resting potentials (around −70 mV), baclofen had no effect on holding current or on input resistance when voltage clamped near resting potential (Fig. 2C), suggesting that the effects on evoked currents are unlikely to be due to a shunting of the postsynaptic potentials/currents. Our conclusion that the effects of baclofen on the synaptic currents were mediated by presynaptic receptors was strengthened by the finding that the ratio of

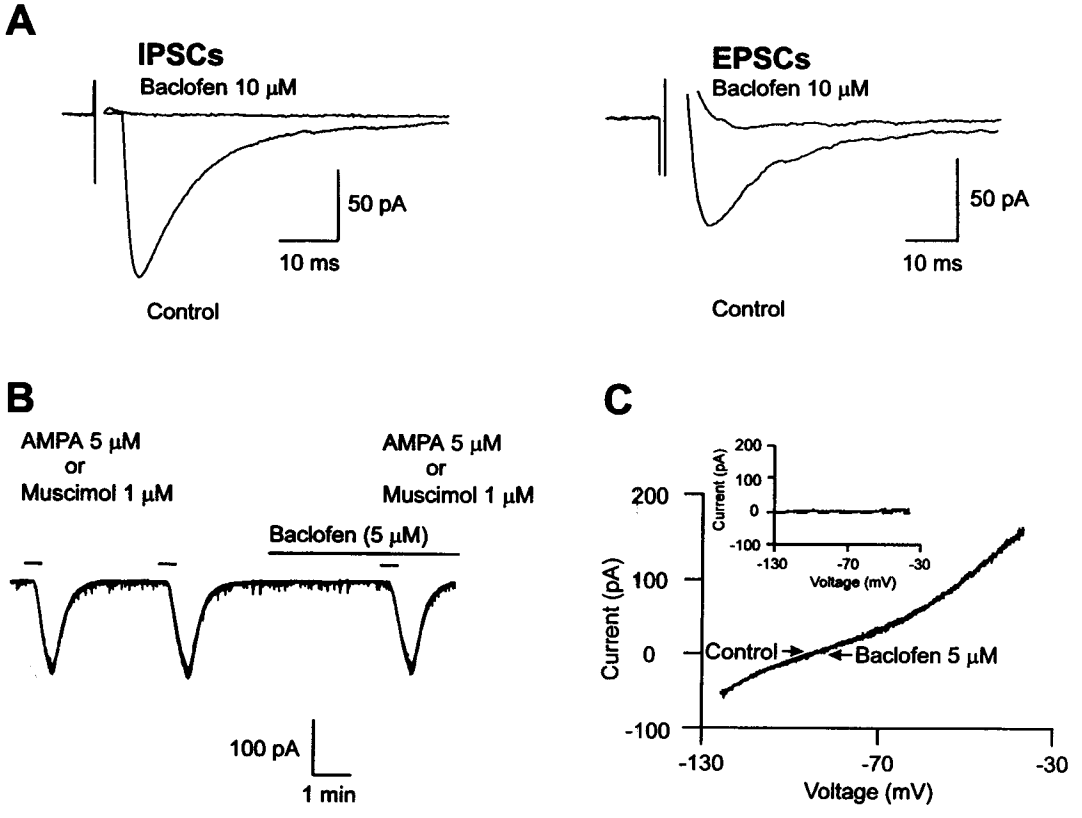

Fig. 2. (A) Reduction of evoked IPSCs (left) and EPSCs (right) by bath application of the $GABA_B$ agonist, baclofen to SON magnocellular neurons voltage-clamped at −80 mV. (B) Either the glutamate receptor agonist, AMPA, or the $GABA_A$ agonist, muscimol activate transient inward currents (mediated by different mechanisms) when applied at times indicated by the short horizontal bars. Application of baclofen during the time indicated by the long horizontal bar did not affect the AMPA or muscimol activated currents. (C) Current evoked by a voltage ramp over a range from −120 to −40 mV is unaltered in the presence of baclofen, illustrating lack of a postsynaptic action of baclofen on currents activated over this voltage range. Insert shows that a subtraction of the current in the presence and absence of baclofen reveals no baclofen-induced current.

the amplitudes of two successive evoked EPSCs or IPSCs to paired stimulations was altered by baclofen. Such an alteration of what is called the 'paired pulse ratio' is thought to be indicative of a presynaptic locus of action (Zucker, 1989).

The role of presynaptic $GABA_B$ receptors in controlling the normal physiology of the SON is still unclear. With the use of a specific $GABA_B$ receptor antagonist, we have been able to demonstrate an action of endogenously released GABA within the SON at these receptors on excitatory terminals, but not at inhibitory terminals. It could be that, in the slice, the highly efficient uptake processes for GABA limit the effectiveness of GABA to act at subsynaptic $GABA_B$ autoreceptors on GABAergic afferents, but not at axo-axonic synapses onto excitatory terminals. Studies in vivo utilizing microdialysis to measure released GABA and glutamate may shed more light on the role of these presynaptic receptors. Initial investigation of the role of central $GABA_B$ receptors in the control of the milk ejection reflex (Voisin et al., 1996) showed that intraventricular injection of baclofen inhibited the reflex, whereas baclofen microinfusion into the SON did not. In addition, microdialysis experiments failed to demonstrate a $GABA_B$ receptor-mediated modulation of basal or potassium-stimulated GABA and glutamate outflow in lactating rats.

A role for the neuropeptides (VP and OT) themselves within the SON in regulating excitability was first postulated when antidromically invaded neurons were found to exhibit a decrease in excitability following the action potential (reviewed in Renaud and Bourque, 1991). This was thought to be due to release of peptide from recurrent collaterals of these neurons. However, intranuclear axon collaterals of magnocellular neurons are now thought to be relatively uncommon (Sofroniew, 1985) and the decrease in excitability is now attributed to a large afterhyperpolarization (Andrew and Dudek, 1984). Nonetheless, magnocellular neurons are capable of releasing their peptide contents within the nucleus in response to depolarizing stimuli either in vitro (Di Scala et al., 1987) or in vivo (Landgraf, 1995),and it appears likely that the neuropeptide release is from dendritic stores (Pow and Morris, 1989). The role of these dendritically released peptides is not yet well understood, but there is some evidence that OT, at any rate, plays a role in facilitating synchronization (Wakerley and Ingram, 1993) and release of the peptide during suckling (Neumann et al., 1994). We have carried out electrophysiological investigations on the actions of the peptides in the SON and have found that bath application of VP and OT had modest effects on the postsynaptic membrane properties of some of the magnocellular neurons. Another action was an inhibition of the EPSC by bath-applied VP (Kombian et al., 1995). Recently, it has also been reported that OT may disinhibit magnocellular SON neurons by depressing tonic GABAergic synaptic inputs via a postsynaptic mechanism (Brussaard et al., 1996). These intranuclear interactions by dendritically-released peptides could be important in reducing afferent excitation of specific cell populations (vasopressinergic?) while allowing others (oxytocinergic?) to be activated.

Extrahypothalamic actions of neurohypophysial peptides

Electrophysiological studies on vasopressin action in the central nervous system have indicated that VP generally has excitatory effects on action potential frequency (Raggenbass et al., 1987; Mo et al., 1992). Intracellular current and voltage studies have shown that a depolarization underlies this excitatory effect; however, the mechanism of this effect appears to vary according to the tissue studied. In rat superior cervical ganglion (Kiraly et al., 1986) and in lateral horn cells (Ma and Dun, 1985), the depolarization is accompanied by an increase in membrane resistance, possibly due to inactivation of a potassium current. In contrast, in neonatal rat facial motoneurons, VP activates a non-inactivating, voltage-dependent sodium current (Raggenbass et al., 1991).

In addition to these direct, postsynaptic effects, mediated by apparent V_1 receptors, there is also evidence that VP may modulate actions of other transmitters. In lateral septum, VP appears to enhance, in a poorly-reversible fashion and after a long latency, excitatory actions of excitatory amino acids (Joels and Urban, 1984; van den Hooff and Urban, 1990) and reduce the inhibitory actions of iontophoretically administered monoamines (Joels

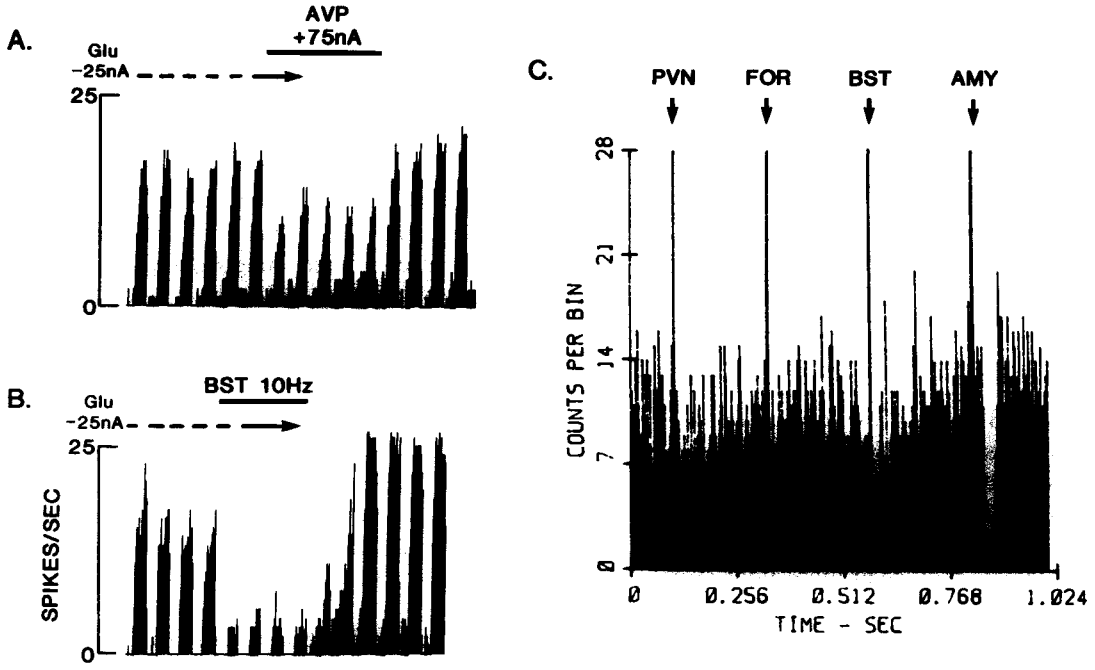

Fig. 3. Arginine vasopressin (VP) reduces glutamate-induced excitation in the ventral septal area, in vivo. (A) Rate meter records of extracellular activity of a cell whose activity is enhanced by intermittent iontophoretic application of glutamate, ejected with a current of −25 nA over 10 s intervals, as indicated by the dashed lines. VP administration from an adjacent micropipette during the time indicated by the horizontal line at a current of +75 nA reduced the glutamate induced activity. (B) Electrical stimulation of the bed nucleus of the stria terminalis (BST) at 10 Hz as indicated by the horizontal line reduces glutamate excitation of the same cell as in (A). (C) Post-stimulus time interval histogram of this cell over 100 repeated sweeps, illustrating cellular responses to electrical stimulation at 1 Hz of the paraventricular nucleus (PVN), fornix (FOR), BST and amygdala (AMY).

and Urban, 1985). In contrast, fast excitatory postsynaptic potentials in the rat superior cervical ganglion are reduced by application of VP (Wali, 1984; Kiraly et al., 1986). With the use of extracellular recordings and iontophoretic or micropressure application of VP, we investigated the actions of the peptide on electrical activity of identified ventral septal area neurons. We identified a subpopulation of neurons upon which we could demonstrate a reversible action of VP to inhibit glutamate, but not acetylcholine induced excitations (Disturnal et al., 1987).The inhibitory action of VP could be mimicked by electrical stimulation of the cell bodies in the bed nucleus of the stria terminalis which give rise to the vasopressinergic innervation of this area (Fig. 3).

Subsequently, we have followed up on these observations by carrying out intracellular, sharp electrode recordings from neurons in slices of the ventral septal area. In approximately one-third of the cells, VP caused a depolarization of 5–18 mV, accompanied by a decrease in input resistance. On a number of other cells, VP had no noticeable effect on either membrane potential, input resistance, spike generation or spike height. However, in these cells, when VP was applied prior to and during glutamate application, the glutamate-induced depolarization and reduction in input resistance was attenuated (Fig. 4). With higher concentrations of glutamate, depolarizations persisted, but small glutamate depolarizations were sometimes totally blocked and the effects of VP often took several minutes after washout to reverse. Thus, in this area of the brain, VP appears to have two

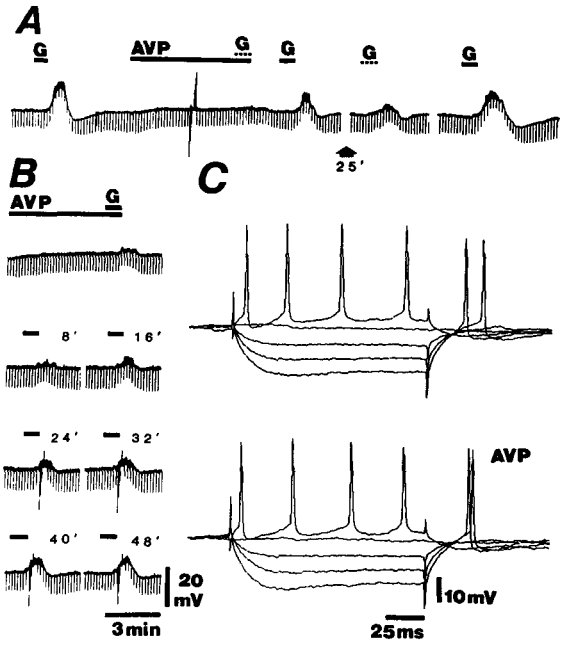

Fig. 4. Intracellular recordings in a ventral septal neuron in vitro illustrating reduction of glutamate (G) action by VP. (A) Voltage recording showing depolarizing responses to G (1 mM; short lines) before and after perfusion with VP (1 μM) revealing a reduction in the depolarization and conductance increase after VP. Response to G (0.5 mM; dotted line) is blocked when co-applied with VP. The second trace in (A) shows the responses to G (1 and 0.5 mM) after 25 min wash. RMP = −63 mV. The periodic hyperpolarizing transients are due to the injection of −0.10 nA current. The hyperpolarizing/depolarizing traces are due to the injection of families of positive and negative currents for construction of current/voltage curves. Action potentials are truncated by the frequency response of the pen recorder. (B) The recovery of G (1 mM) depolarization after VP application is shown with 8 min time intervals in a different cell. RMP = −60 mV; time and voltage scale apply also for (A). (C) Voltage recordings from the cell in (A) are shown during current pulses of +0.10, 0.0, −0.10, −0.20 and −0.30 nA before (upper traces) and during (lower traces) incubation with VP for 3 min.

distinct actions: one to directly alter postsynaptic membrane properties and a second to interfere with the action of excitatory amino acid transmitter at a postsynaptic site.

Additional evidence that VP can interact with excitatory transmission has been obtained in a brainstem nucleus, the parabrachial nucleus. This nucleus is an important relay nucleus passing visceral afferent information (i.e., from the vagus and from spinal pathways) onto higher central nervous system areas such as the thalamus and the amygdala. In slice preparations of this area, afferent perinuclear stimulation elicits EPSCs that are glutamate mediated (Zidichouski and Jhamandas, 1993). Perfusion of VP onto the slice causes little change in postsynaptic membrane properties, nor does it interfere with the action of exogenously applied glutamate agonists. However, the EPSC is reversibly attenuated in a dose-dependent fashion (Fig. 5). Thus, it would appear that VP is acting on receptors at presynaptic terminals in the parabrachial nucleus, possibly in a manner similar to that discussed earlier in the SON. Such an action could account for VP's action to elevate blood pressure when it is given intraventricularly or into a number of limbic and autonomic nuclei.

Conclusion

From an electrophysiological standpoint, VP has numerous actions within the central nervous system. Most of the actions have been shown to be mediated by a receptor resembling the V1 subtype. Thus, it may be that the identical receptor is coupled to a number of different intracellular

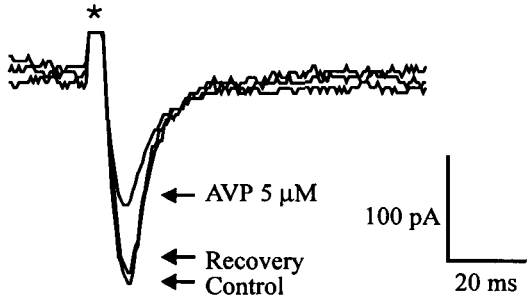

Fig. 5. EPSCs evoked in the parabrachial nucleus, in vitro, by stimulation of glutamatergic afferents at the time illustrated by the asterisk (shock artefact truncated). VP causes a reversible attenuation of the EPSC with little effect upon postsynaptic membrane properties (holding potential = −65 mV).

signal transduction mechanisms. With respect to OT, whose action is also mediated by receptors resembling the uterine OT receptor (Muhlethaler et al., 1983; Tolchard and Ingram, 1993), both pre- and postsynaptic actions have been identified. A dramatic action of these peptides upon intracellular calcium levels has also been identified (Lambert et al., 1994; Dayanithi et al., 1996), and it will be important to determine how these effects are related to the membrane and synaptic actions described above. In addition to this action of dendritically released peptides upon afferent and intrinsic excitability of magnocellular neurons, other afferent transmitters interact to 'gate' afferent inputs. Examples of this were provided by our studies showing that GABA receptors upon presynaptic excitatory and inhibitory afferent terminals inhibit transmitter release. The physiological relevance of these latter actions are yet incompletely understood and require further in vivo studies.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 3.2.5

Electrophysiological effects of oxytocin within the bed nuclei of the stria terminalis: Influence of reproductive stage and ovarian steroids

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The bed nuclei of the stria terminalis (BNST) is a target site for the central actions of oxytocin (OT) in promoting behavioural and neuroendocrine responses involved in female reproduction, and binding studies suggest that OT sensitivity may be modulated over the peripartum period. Electrophysiological recordings from brain slices in vitro showed that OT sensitivity of BNST neurones is relatively low in late pregnancy, but is high during lactation. In vivo studies over the immediate peri-partum period revealed that although BNST neurones can be excited by i.c.v. OT at day 22 of pregnancy, there is a 5–10 min delay in their response which is not present in lactation. This delay can be reversed by naltrexone, or lesioning the stria terminalis, and may involve an inhibitory opioid input to the BNST from the amygdala. Examination of the role of steroids in regulating OT responses of BNST neurones showed that oestradiol pre-treatment in late pregnant ovariectomized rats increased OT excitation of BNST neurones in vitro, and a similar result was observed with in vivo recordings. Progesterone also augmented OT excitation of BNST neurones in vitro, but no such effect was observed in vivo. This difference could indicate that an additional effect of progesterone is to potentiate extraneous inhibitory inputs to the BNST, or may reflect the ability of this steroid to suppress OT sensitivity by a direct membrane action. Changes in the response of BNST neurones to OT may have functional implications for the action of central OT in facilitating the neuroendocrine milk-ejection reflex (i.e. increasing milk-ejection frequency), an effect which first appears at around day 3 of lactation. Studies involving steroid treatment of late pregnant ovariectomized rats showed that this facilitatory mechanism can be induced to appear early (i.e. on day 22 of pregnancy) by oestradiol, but not progesterone treatment. Collectively, these results support this view, that the action of OT in the BNST is regulated by the changing levels of steroids towards the end of pregnancy, thereby ensuring appropriate neuroendocrine responses necessary for motherhood.

Introduction

The bed nuclei of the stria terminalis (BNST) are a collection of limbic nuclei located between the hypothalamus and septal region, and are closely related to the amygdala, their major source of afferent input (Weller and Smith, 1982). On the basis of cytoarchitecture, the BNST have been divided into major anterior and posterior divisions, with the former further subdivided into lateral, dorsal and ventral areas (Ju and Swanson, 1989). Within the lateral area of the anterior division of the BNST are located the oval and juxtacapsular nuclei; the posterior division of the BNST contains the principal encapsulated nucleus. The dorsal area of the anterior division corresponds to the medial BNST described by De Olmos et al. (1985), and on the basis of its connectivity with preoptic and ventromedial hypothalamic nuclei, as well as functional studies, this region has been implicated in neuroendocrine regulation and sexual behaviour. The lateral area of the anterior division of the BNST, on the other hand, is mainly concerned with autonomic

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and electrolyte control, and has major reciprocal connections with the brain-stem and pontine brachial nucleus (McKellar and Loewy, 1982). All the BNST receive dense noradrenergic innervation from the medulla (Swanson and Cowan, 1979), but this noradrenergic input is particularly strong to the ventral area of the anterior BNST (Sofroniew, 1983; Woulfe et al., 1990; Terenzi and Ingram, 1995).

Initial evidence concerning the role of the BNST as a target site for the central actions of oxytocin (OT) came from binding studies which localized OT binding to the dorsal regions of the BNST (Freund-Mercier et al., 1987). In a later more detailed autoradiographic analysis by Krémarik et al. (1991), using a highly selective ^{125}I-labelled OT antagonist, two distinct areas of OT binding were demonstrated: the principal encapsulated nucleus and the oval nucleus. Weak labelling was also found in anterior–lateral and anterior–dorsal areas surrounding the oval nucleus. Kremarik et al. found that OT binding in the oval nucleus was not oestrogen regulated, contrasting with binding in the principal encapsulated nucleus which appeared to be oestrogen dependent. It is highly likely that these BNST OT binding sites represent OT receptors, a conclusion supported by in situ localisation of mRNA encoding a sequence similar to the human uterine OT receptor (Yoshimura et al., 1993).

The ability of OT to excite BNST neurones has been demonstrated in our laboratory by in vitro recordings in brain slices obtained from lactating rats (Ingram et al., 1990). Examples are shown in Fig. 1. Approximately 50% of spontaneously active neurones within the BNST are excited by OT, and the receptors involved have the properties of uterine-type OT receptors, in that they are affected with similar potency by OT or by the specific OT-receptor agonist [Thr^4,Gly^7]OT (TGOT). They can also be antagonized by the broad spectrum antagonist [d(CH$_2$)$_5$,D-Tyr(OEt)^2,-Val^4,Cit^8]vasopressin. BNST neurones which respond to OT are also excited by vasopressin administration, but with on average 5-fold less potency (Ingram et al., 1995) which is consistent with involvement of OT receptors. Interestingly, the BNST region in which most OT-sensitive neurones are located does not correspond to areas of highest OT binding density (Fig. 1). Electrophysiological studies in vivo, using anaesthetized lactating rats, showed that approximately 25% of neurones recorded in the ventrolateral septum-BNST region were excited by an i.c.v. injection of 2.2 ng (1 mU) OT, and gave a prolonged increase in firing, lasting at least 15 min (Lambert et al., 1993). Many of the neurones which were excited by OT displayed a distinctive background firing pattern with repetitive clusters of high frequency spikes, suggesting that they belonged to a particular sub-population of cells. Some OT-sensitive neurones of this type showed cyclical activity correlated with the intermittent activation of the milk-ejection reflex.

Supporting evidence for an important role of the septal region as a target site for central OT has come from studies employing push-pull perifusion or microdialysis which has demonstrated increases in OT concentrations within the limbic system during stimuli such as parturition and suckling both in rats (Landgraf et al., 1992) and sheep (Kendrick et al., 1992). What is the normal source of this OT increase? Although injection of OT into the ventricular system will excite BNST neurones (Lambert et al., 1993; Ingram et al., 1995), there is currently little evidence that this is the natural route for the action of OT. More likely, the BNST receives a direct oxytocinergic innervation, possibly derived from OT neurones lying in the rostral hypothalamus, distinct from the magnocellular system. Groups of OT neurones have been reported in the medial proptic area (anterior and lateral commissural nucleus, periventricular region) which increase their levels of OT mRNA and OT immunoreactivity during parturition or lactation (Caldwell et al., 1987; Jirikowski et al., 1989; Brooks et al., 1990; Broad et al., 1993). Studies in the lactating rat (Ingram and Moos, 1991) have demonstrated OT-immunoreactive varicose fibres within the BNST and ventrolateral septum which arise from a distinct group of OT-containing neurones in the anterior commisural nucleus. Furthermore, stimulation of this pathway in an oblique tissue slice in vitro results in excitation of OT-sensitive BNST neurones that can be reversibly blocked by an OT receptor antagonist.

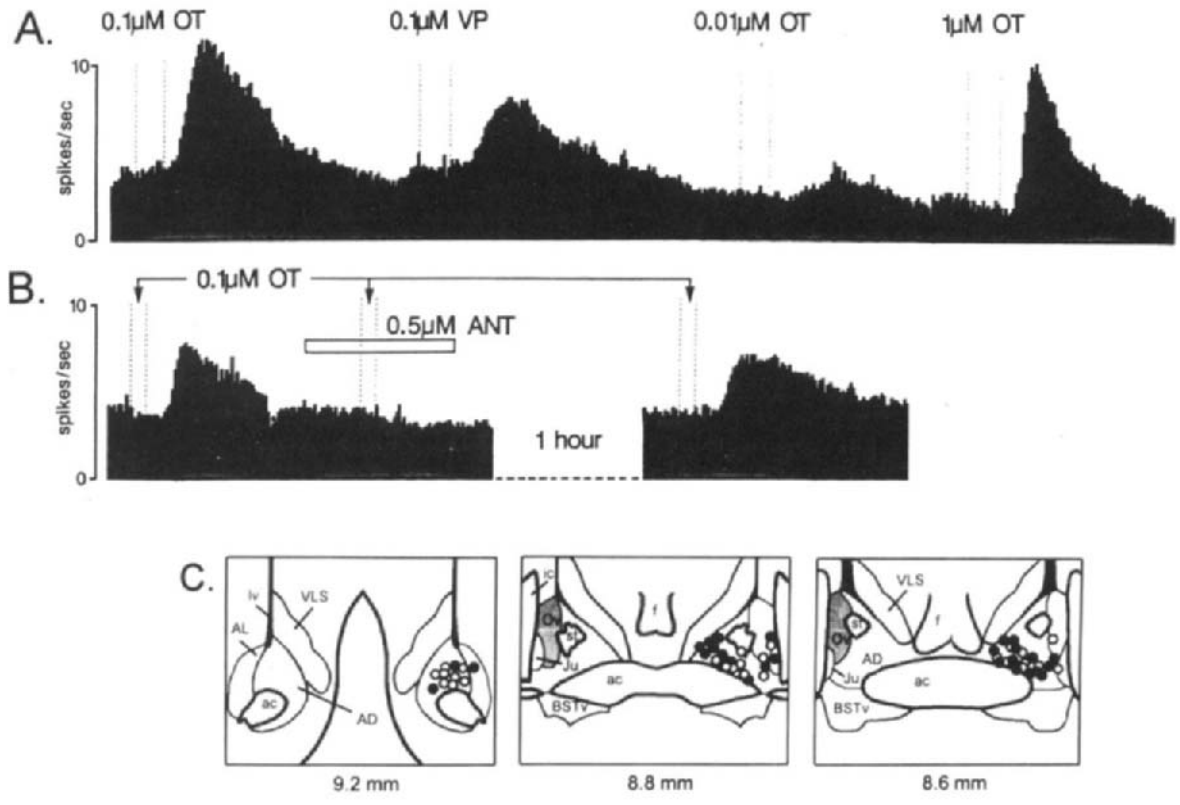

Fig. 1. Neurones in the BNST are excited by oxytocin. (A) Firing rate of a BNST neurone in vitro during application of oxytocin (OT) to the perfusate (brain slice from a lactating rat). Note vasopressin (VP) was less effective than OT. (B) BNST neurone during application of OT and the effect of the receptor antagonist [d$(CH_2)_5$, d-Tyr$(OEt)^2$,Val^4,Cit^8]VP (ANT). (C) Coronal sections through the BNST of the rat according to the stereotaxic atlas of Paxinos and Watson (1986) and nomenclature from Ju and Swanson (1989). On the right-hand side of each diagram, ● indicate position of OT-sensitive neurones, and ○ represent OT-insensitive neurones. On the left-hand side is indicated the relative density of oxytocin binding in the different regions, based on Krémarik et al. (1991). Abbreviations: ac, anterior commissure; AD, anterodorsal subnucleus of the BNST; AL, anterolateral subnucleus of the BNST; BSTv, ventral part of the BNST; f, fornix; ic, internal capsule; Ju, juxtacapsular subnucleus of the BNST; lv, lateral ventricle; Ov, oval subnucleus of the BNST; st, stria terminalis; VLS, ventrolateral septum.

Changes in the electrophysiological responses of BNST neurones to OT in different reproductive states

Central OT binding sites, like their counterparts in peripheral tissues such as uterus and mammary gland, undergo substantial changes in density between different reproductive states. Autoradiographic studies demonstrated a significant increase in OT binding within the BNST on day 1 postpartum relative to post-lactating cyclic females, apparently resulting from an increase in binding sites rather than a change in affinity of OT receptors (Insel, 1986, 1990). The increase in OT binding in the BNST begins in mid-pregnancy and lasts through until at least day 6 of lactation (Insel, 1986, 1990). The BNST also shows changes in OT binding during the oestrous cycle (Krémarik et al., 1991), with levels peaking at the time of oestrus. By analogy with what has been reported for the ventromedial hypothalamic nucleus and amygdala (Bale and Dorsa, 1995), it is probable that these changes in OT binding in the BNST result from increased expression of messenger RNA for the OT receptor.

An important question is whether changes in OT

binding sites in the BNST in different reproductive states are accompanied by a parallel increase in the electrophysiological responses to OT. We have examined this question by comparing responses of BNST neurones with the specific OT agonist, TGOT, in brain slices taken from rats in different reproductive stages (mid-pregnancy, early- and mid-lactation, and after weaning) (Ingram and Wakerley, 1993). Recordings were taken from the anterodorsal subnucleus of the BNST and each neurone was challenged with two doses (0.1 and 0.01 mM) of TGOT. Whilst the numbers of BNST neurones which responded to TGOT (40–44%) across the different groups remained constant, there were marked changes in the magnitude of the responses. Thus BNST neurones in slices taken in early- or mid-lactation showed responses that reached a higher peak and lasted longer than neurones in slices from the mid-pregnant and weaned groups (Fig. 2). Differences were even more evident with the lower dose of TGOT, which evoked responses only in slices from the two lactating groups. These in vitro results indicate that lactation is associated with an increase in the OT sensitivity of BNST neurones, and these results are broadly in line with the reported changes OT binding sites (Insel, 1990). One notable difference is that the electrophysiological results indicate that the elevated OT sensitivity is maintained at least until mid-lactation, whereas the increase in OT binding seems to be evident only over the first few days after birth. This discrepancy may relate to additional changes in post-receptor transduction mechanisms which help to maintain a high OT sensitivity during lactation.

Whilst in vitro electrophysiological studies can provide important information about changes in the direct sensitivity of BNST neurones to OT, it should be remembered that, under normal physiological circumstances, the response to OT will also be fashioned by the additional influence of afferent circuitry impinging on the BNST. Thus we have also examined changes in the response of BNST neurones to OT over the peri-partum period by undertaking recordings in vivo (Housham et al., 1997). These experiments were performed on urethane-anaesthetized rats which were taken on days 19 or 22 of pregnancy, or on day 5 of lactation, and each BNST neurone was tested for its response to a single i.c.v. injection of 1.1 ng OT. Individual examples illustrating the results are shown in Fig. 3. Somewhat surprisingly, we found no significant difference in the amplitude of the responses across the different stages, although the peak firing rates during the responses tended to rise from day 19 (range 4–5 spikes/s) to day 22 of pregnancy (6–7 spikes/s) and decline again by day 5 of lactation (4–5 spikes/s). However, one important group difference to emerge from these experiments was that, whereas the response to OT on day 19 of pregnancy occurred after only a short latency of around 5 min, the response on day 22 was significantly delayed with the latency almost doubled to over 10 min (Fig. 3). This delay was no longer evident by day 5 of lactation. A consequence of the delayed response at day 22 of pregnancy was that overall activity in a 15-min period following OT injection was substantially reduced compared with the other stages.

The earlier in vitro results suggested that OT sensitivity of BNST neurones increased during the peripartum period, but the recordings in the intact animals show that the excitatory effect of central OT on BNST neurones may be modulated by additional factors near the time of parturition, which prevent full expression of the OT response. It would seem unlikely that the observed delay in excitation following i.c.v. OT on day 22 of pregnancy related to any pharmacokinetic differences in the way OT diffused through the brain. Differences in the post-receptor mechanism, which probably operates over a time course of seconds rather than minutes, would also seem unlikely. More probably, this delay results from activation of inhibitory afferents (absent in a brain slice) which suppress the actions of OT on the BNST. Oxytocin-sensitive neurones in the BNST are also sensitive to opioid peptides (Ingram et al., 1995), and stimulation of the stria terminalis in in vitro tissue slices causes a naloxone reversible inhibition of BNST neuronal activity (Sawada and Yamamoto, 1981), consistent with this being a major inhibitory input to the BNST. We (Housham et al., 1997) have recently obtained evidence for the involvement of this pathway in delaying the OT response of BNST neurones at day 22 of pregnancy. Thus, the delay is abolished

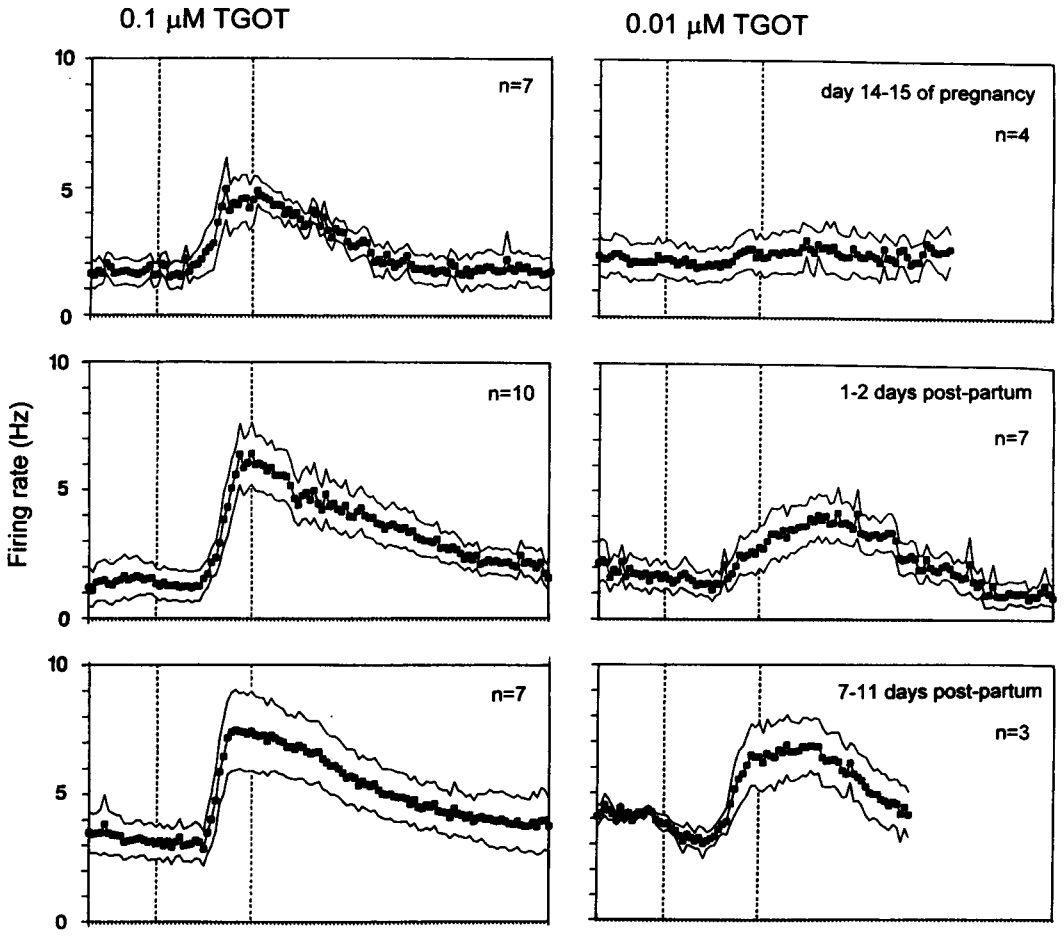

Fig. 2. Sensitivity of BNST neurones to oxytocin increases between pregnancy and lactation. Responses to the OT agonist [Thr^4,Gly^7]OT (TGOT) for groups of BNST neurones in brain slices taken from rats at days 14–15 of pregnancy, days 1–2 post-partum and days 7–11 post-partum rats. Graphs show mean ± SEM firing rate in 10 s bins during the response to a 4 min pulse (vertical lines) of 0.1 or 0.01 μM TGOT.

by treatment with the opiate antagonist, naltrexone. Naltrexone did not affect the baseline firing rate of BNST neurones, suggesting that the inhibitory opioid input is not tonically active (at least in the anaesthetized animal) but is probably activated following i.c.v. OT injection. Since the BNST is innervated by enkephalin-containing fibres from the amygdala via the stria terminalis (Uhl et al., 1978; Rao et al., 1987), we have extended these studies by attempting to abolish the delay in the OT response of BNST neurones in day 22 pregnant rats by lesioning this pathway (Housham et al., 1997). Lesions of the stria terminalis reduced the latency of onset of the excitatory response to OT to values comparable with those seen in day 19 pregnant or day 5 lactating animals.

The amygdala also contains OT binding sites (Freund-Mercier et al., 1987; Tribollet et al., 1990; Krémarik et al., 1991, 1993, 1995; Patchev et al., 1993; Condés-Lara et al., 1994), providing a potential mechanism whereby the opioid pathway could be activated by i.c.v. injection of OT. Presumably this input to the BNST itself undergoes adaptations during the transition from pregnancy to

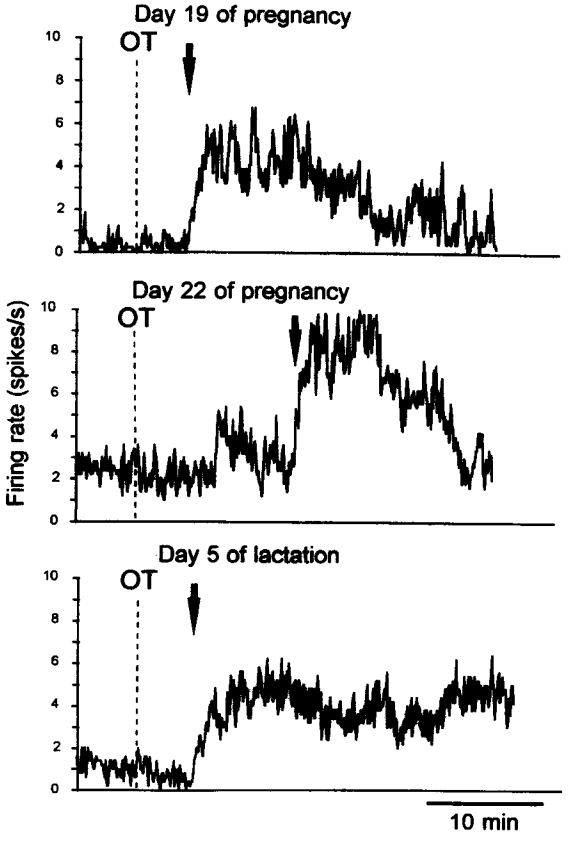

Fig. 3. Neurones in the BNST show a delayed response to OT on the day of parturition. Typical response profiles of BNST neurones recorded in urethane-anaesthetized lactating rats following i.c.v. injection of OT (1.1 ng, at dotted line) on different days in the peri-partum period. The arrow indicates the time of the onset of the response.

lactation, although this does not seem to involve changes in the OT binding in the amygdala (Insel, 1990).

Role of steroids in regulating electrophysiological responses of BNST neurones to OT

The ability of ovarian steroids to modulate OT binding density within the BNST (Insel, 1986; Tribollet et al., 1990; Krémarik et al., 1991, 1995; Patchev et al., 1993) and other forebrain structures (Coirini et al., 1992) is well established. The changing levels of steroids in late pregnancy might therefore provide a basis for alterations in the response of BNST neurones to OT during the transition from pregnancy to lactation. The peripartum period is associated with a gradual rise of oestradiol from day 16 of pregnancy up to the time of birth, and there is a further transitory rise of oestradiol 24 h post-partum (Yoshinaga et al., 1969; Garland et al., 1987). Progesterone, on the other hand, shows a peak at around day 19–20 of pregnancy and then declines rapidly following luteolysis (Garland et al., 1987).

The possible role of oestradiol in the peripartum changes in OT response of BNST neurones has been studied in our laboratory using both in vivo and in vitro electrophysiological studies (Fig. 4). Rats were ovariectomized and hysterectomized on day 19 (in vivo studies) or day 20 (in vitro studies) of pregnancy and treated daily with 10 μg oestradiol. On the day equivalent to day 22 of pregnancy, the response of BNST neurones to OT was tested either in vivo using i.c.v. injection of OT under urethane anaesthesia, or in vitro using perifusion of slices with TGOT. Consistent with reports of oestradiol increasing OT binding (Insel, 1986; Krémarik et al., 1991, 1995; Patchev et al., 1993), both sets of experiments clearly showed that oestradiol treatment significantly augmented the response of BNST neurones to OT or TGOT compared with oil-treated controls (Fig. 4). The results for the two approaches were very similar, with a more or less doubling of the average firing rate achieved at the peak of the response compared to controls (range 5–6 vs. 2–3 spikes/s in the in vivo studies; 6–7 vs. 3–4 spikes/s in the in vitro studies). The in vivo recordings showed no sign that oestradiol treatment was associated with any delay in the OT response (see previous section of this review). In fact, the only notable difference in the two sets of data was that the recordings in the intact animal did not reveal any effect of oestradiol on the numbers of OT sensitive BNST neurones, whereas in the in vitro recordings this parameter was increased from 18 to 54%. The reason for this difference is presently unclear.

Using an experimental protocol similar to that for examining the effects of oestradiol (see above) we have found that, in in vivo studies on the intact brain, progesterone pre-treatment (10 mg/day) had no effect on the response of BNST neurones to OT. However, in in vitro studies, similar progesterone

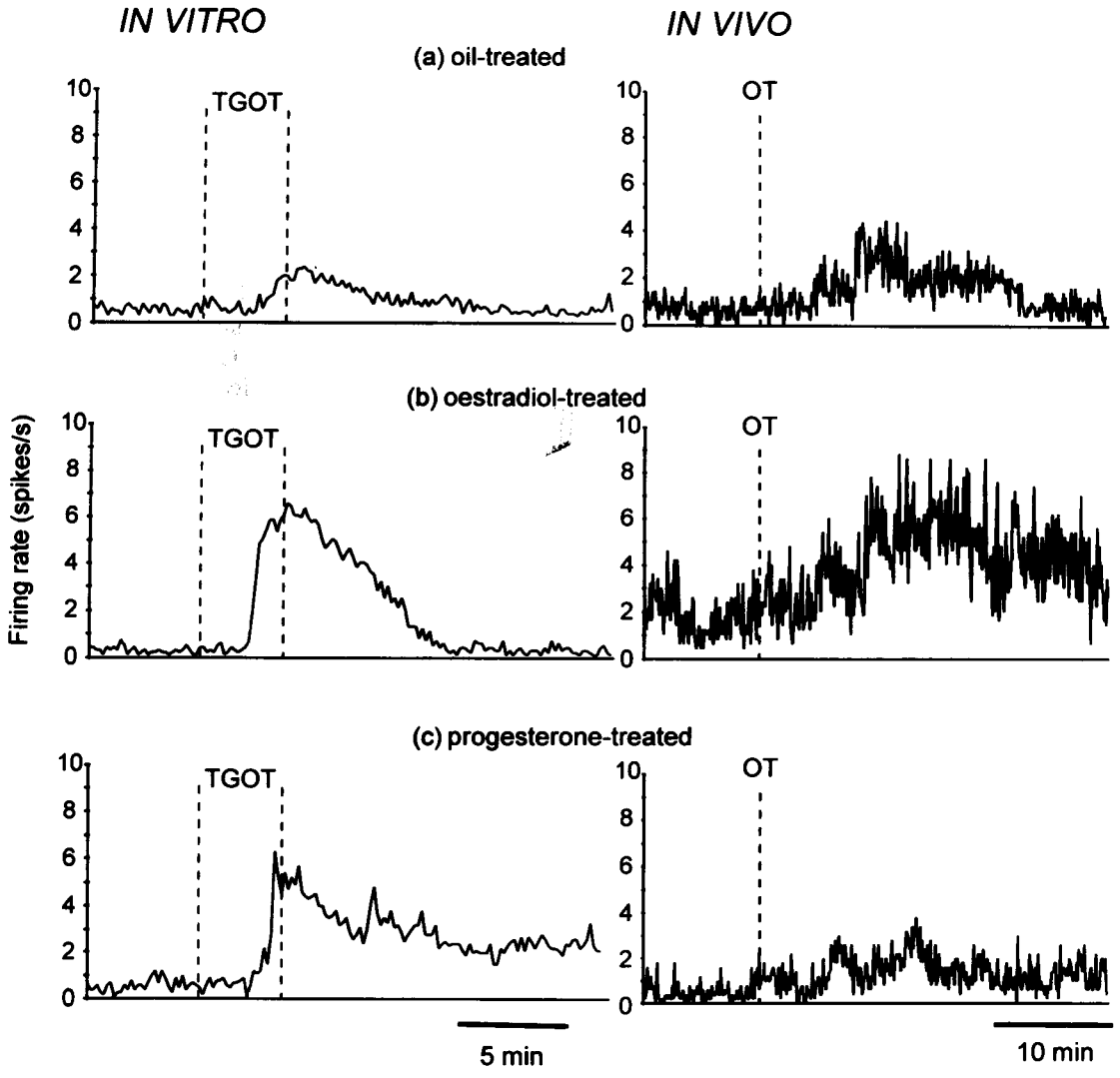

Fig. 4. Gonadal steroids modulate the response of BNST neurones to central OT. Response profiles of BNST neurones recorded in vivo (urethane-anaesthetized rats) or in vitro (hypothalamic slices) following ovariectomy and oil treatment (control), oestradiol treatment, or progesterone treatment. Neurones in vivo were tested with i.c.v. injection of OT (1.1 ng), neurones in vitro with a 4 min pulse of [Thr^4,Gly^7]OT (TGOT, 10^{-7} M). See text for details.

pre-treatment did augment the response to OT (Fig. 4). In fact, average values for the peak firing rates achieved during TGOT perifusion in slices from progesterone-treated rats were similar to those seen after oestradiol, although there was noticeably more variability in the amplitude of responses recorded from slices taken after progesterone treatment. Unlike oestradiol, progesterone had no effect on the numbers of responsive cells. Explaining the difference between these in vivo and in vitro results following progesterone treatment is compounded by the lack of consistency with regard to the reported effects of this steroid on OT binding. Thus Patchev et al., (1993) reported that proges-

terone increased OT binding within the BNST, whereas others (Krémarik et al., 1995) have found that OT binding in the telencephalon (including the BNST) is unaffected by progesterone. The in vitro results would certainly support a role for progesterone, like oestradiol, in increasing OT sensitivity in the BNST, and it would be useful to examine this further with combinations of the two steroids.

Assuming that progesterone can augment OT sensitivity of BNST neurones, as the in vitro results would suggest, why was no effect observed in the in vivo studies? One speculative explanation may be that progesterone treatment produces a situation reminiscent of that discussed in relation to in vivo observations on the OT response of BNST neurones on day 22 of pregnancy (see previous section). In other words, progesterone may not only augment the OT sensitivity of BNST neurones, but also potentiate an inhibitory opioid input which, in the intact brain, restrains their response. In support of this, progesterone produces multiplicative effects within the brain, including a rise in opioid tone (Kalra, 1993). An alternative explanation for the difference between the in vitro and in vivo results following progesterone treatment is provided by a recent study by Grazzini et al. (1998) showing that progesterone can disrupt ligand binding of OT receptors through a rapid membrane effect. The maintained high level of circulating progesterone in the in vivo experiments would explain the absence of any increased electrophysiological response to OT compared with control animals, whereas the absence of progesterone in vitro would allow full OT (or TGOT) sensitivity to be expressed.

Functional implications of steroid modulation of OT responses in BNST neurones

In the context of female reproduction, the actions of OT within the BNST have been implicated in several important functions, most notably in the promotion of sexual and maternal behaviour (Insel, 1992). In both cases, the stimulatory effects of OT require an appropriate steroidal background, underlining the potential importance of steroid modulation of OT sensitivity of BNST neurones described in the previous section.

Our own studies concerning the functional consequences of changes in the central actions of OT have focused on the role of the BNST in facilitation of the neuroendocrine milk-ejection reflex. This facilitation can be evoked by i.c.v. injection of OT, and is manifest as an increase in the frequency and amplitude of suckling-evoked bursting activity in the magnocellular OT neurones (see Freund-Mercier and Richard, 1984; Richard et al., 1988 for reviews of this effect). There is extensive evidence based on lesioning (Ingram et al., 1995), microinjections of OT (Moos et al., 1991), and electrophysiological recording (Lambert et al., 1993), that the BNST co-ordinates this facilitatory effect.

Electrophysiological recordings of supraoptic neurosecretory neurones during suckling have shown that the facilitatory mechanism undergoes dynamic changes in different reproductive states, being weak or absent during late pregnancy and after weaning, but powerful in mid-lactation (Hughes et al., 1993; Jiang and Wakerley, 1995) (Fig. 5). Analysis of the facilitatory effect on each successive day post-partum has revealed that the mechanism underlying this response first becomes operational on day 3 of lactation (Housham and Ingram, 1995). This is perhaps surprising in view of our in vitro electrophysiological studies suggesting that the postpartum increase in the sensitivity of BNST neurones to OT is already well established by day 2 of lactation (Ingram and Wakerley, 1993).

A clue as to why no facilitation is observed earlier than day 3 may be found in the results from our in vivo studies showing that between birth and the first 3 days of lactation, the response of BNST neurones following i.c.v. OT is delayed by 5–10 mins compared with later in lactation (Fig. 3). The initial period following OT administration is critical for allowing full expression of the facilitation since, after this time, arousal of the EEG tends to inhibit the milk-ejection responses (Wakerley et al., 1989). The delayed response of the BNST neurones to OT, present over the first few days of lactation may, therefore, explain why facilitation is absent at this time. One advantage of programming the facilitation such that it does not appear until around day 3 of lactation may be in avoiding inappropriate activation of limbic circuits concerned

with potentiating pulsatile OT release until well after birth has been successfully accomplished.

The important influence of ovarian steroids on the electrophysiological response of BNST neurones to OT (see previous section) may provide an obvious explanation of how the mechanisms concerned with facilitation of the milk-ejection reflex can be programmed at the start of lactation. Evidence in favour of such a role of steroids in programming facilitation has been obtained in our laboratory by evaluating the facilitatory response to i.c.v. OT in day 22 pregnant rats which had been ovariectomized 2 days previously and pre-treated with oestradiol or progesterone (Jiang and Wakerley, 1997) (Fig. 6). Oestradiol (5 μg/day) was found to advance the facilitation of the milk-ejection reflex so that it was already present by day 22 of pregnancy. Similar results were obtained in ovariectomized hysterectomized animals pre-treated with oestradiol, so this finding was unrelated to the premature parturition induced by oestradiol, but would be consistent with the steroid bringing about an increase in OT action within the BNST. Identical studies have also examined the effects of progesterone pre-treatment (5 mg/day) on the facilitatory response. Interestingly, progesterone was found to completely alter the response to i.c.v. OT, such that there was a decrease in the bursting of magnocellular supraoptic neurones and milk-ejection frequency following injection (Fig. 6). This effect requires further investigation but the absence of facilitation after progesterone treatment is consistent with our earlier in vivo electrophysiological results indicating that this steroid does not enhance OT-induced excitation of BNST neurones.

Since oestradiol treatment can increase the response of BNST neurones to OT as well as induce other mechanisms necessary to enable central OT to facilitate the milk-ejection reflex, it is reasonable that this steroid has a major role in programming the appearance of the facilitatory mechanism at the start of lactation. However, in view of the fairly rapid effect of oestradiol in inducing the facilitatory mechanism within 2 days of treatment (Jiang and Wakerley, 1997), it is perhaps hard to explain why under natural conditions facilitation does not appear earlier than day 3 of lactation. It is possible that induction of facilitation by the gradual rise of

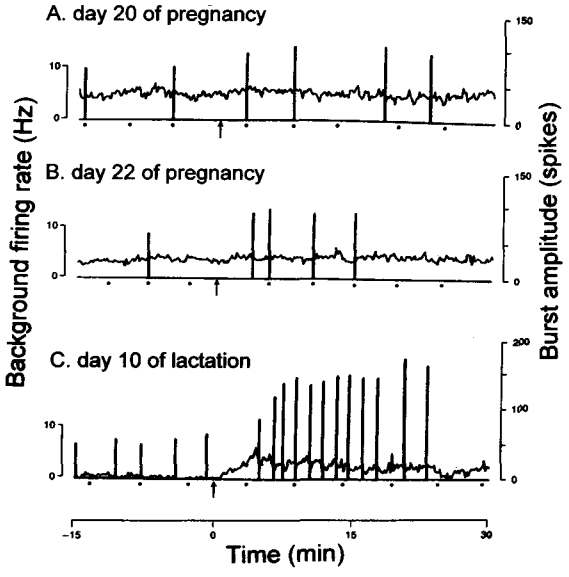

Fig. 5. The facilitatory effect of central oxytocin is established during lactation. Electrophysiological recordings from oxytocin neurones in urethane-anesthetized rats to show the facilitatory effect of i.c.v. OT (2.2 ng, arrow) on bursting activity of supraoptic neurones in the different reproductive states indicated. Each of these records was made during suckling combined with cervico–vaginal probing undertaken every 5 min (dots) to evoke additional bursts. Background activity (left scale) is indicated by the continuous line and burst amplitude is indicated by the vertical bars (right scale).

endogenous oestradiol occurring in late pregnancy may operate over a much longer time course than when oestradiol is rapidly increased by giving exogenous steroid. The time course of the effects of endogenous oestradiol may also be influenced by the residual effect of progesterone which, from the replacement studies described above, may tend to oppose the appearance of facilitation.

A second more substantial rise of oestradiol occurs in association with the post-partum oestrus (Yoshinaga et al., 1969; Garland et al., 1987), and this might also provide a further cue for the appearance of the facilitatory response to i.c.v. OT. However, we have found that the facilitatory response to i.c.v. OT tested on day 3 of lactation was unaffected either by ovariectomy on day 21 of pregnancy (Fig. 7) or by administration of the anti-oestrogen, tamoxifen, starting on day 22 of preg-

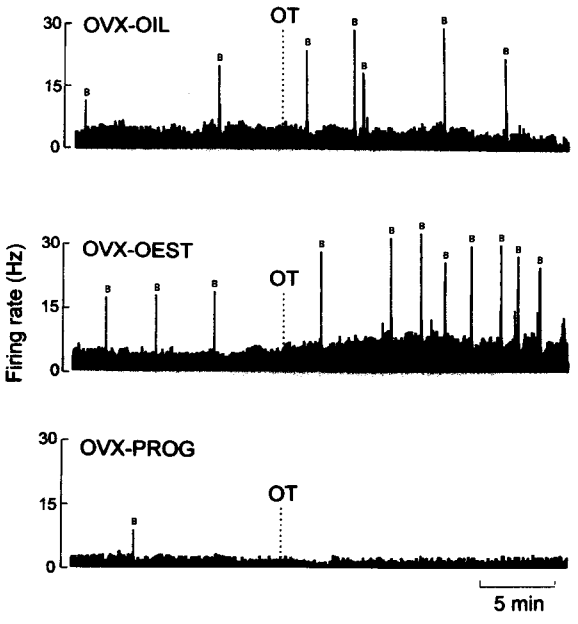

Fig. 6. Effect of steroid pre-treatment on bursting activity of OT neurones in the supraoptic nucleus during suckling. Oestradiol (5 μg/day) but not progesterone (5 mg/day) pre-treatment for 2 days following ovariectomy at day 20 of pregnancy causes establishment of the facilitatory effect of central OT on the milk-ejection reflex on day 22 of pregnancy. The histograms shows firing rates (3 s bins) of individual OT neurones recorded from ovariectomized (OVX) rats pre-treated with oil-vehicle (oil), oestradiol (oest) or progesterone (prog). Records span 15 min before and 20 min after i.c.v. administration of OT (2.2 ng). High frequency bursts associated with milk ejection are marked by 'B'.

nancy (Housham and Ingram, 1995). Thus, it would seem likely that the programming of OT facilitation of the milk-ejection reflex involves pre-partum rather than post-partum exposure to oestradiol.

Hypothesis: Steroidal modulation of OT action within the BNST enables appropriate neuroendocrine responses required for lactation

It has been proposed (see McCarthy, 1995) that the action of central OT in promoting reproductive behaviours may result from a generalized anxiolytic effect which reduces the level of emotional stress which would otherwise disrupt the behaviour. In support of this, Fahrbach et al. (1986) found that virgin rats only showed increased maternal behaviour in response to OT if they had very recently been habituated to the testing cage so that they were still in a stressed condition. It may also be significant that the ability of OT to increase female sex behaviour is best observed when the tests are undertaken in a novel testing arena, with the female being exposed to an unfamiliar individual. McCarthy (1995) has pointed out that OT may enable expression of appropriate maternal or sexual behaviour by reducing anxiety arising from an usual social environment (i.e. new-born pups or a strange male) which might otherwise evoke a fear response and disrupt the behaviour. Adding weight to this argument, an anxiolytic effect of OT has been demonstrated in an elevated plus maze (McCarthy and Goldman, 1994; Windle et al., 1997a). In common with other central effects of OT, this anxiolytic action has been found to be dependent upon oestradiol pre-treatment (McCarthy and Goldman, 1994). It has yet to be established whether progesterone can modulate the anxiolytic effect of OT.

The idea of OT exerting its central effects on reproductive behaviours through suppression of anxiety can be extended to the central action of this peptide in the milk-ejection reflex. It is well known that emotional stress inhibits the milk-ejection reflex (Wakerley et al., 1994), so that even under the most careful experimental conditions there may sufficient stress to slow the frequency of pulsatile OT release evoked by suckling. Administration of i.c.v. OT may alleviate this stress inhibition, so enabling a sudden increase in the frequency of OT pulses. In this regard, it may be functionally important in relation to the milk-ejection reflex that lactating rats show a marked suppression of the normal response to stress, and this may be related to upregulation of central OT pathways (Lightman et al., 1997). It has recently been found that lactating rats show no neuroendocrine response to noise stress, whereas in virgin rats the same stimulus produces increased ACTH and corticosterone levels (Windle et al., 1997b). However, the response of virgin rats could be markedly attenuated by i.c.v infusions of low doses (1–10 ng/

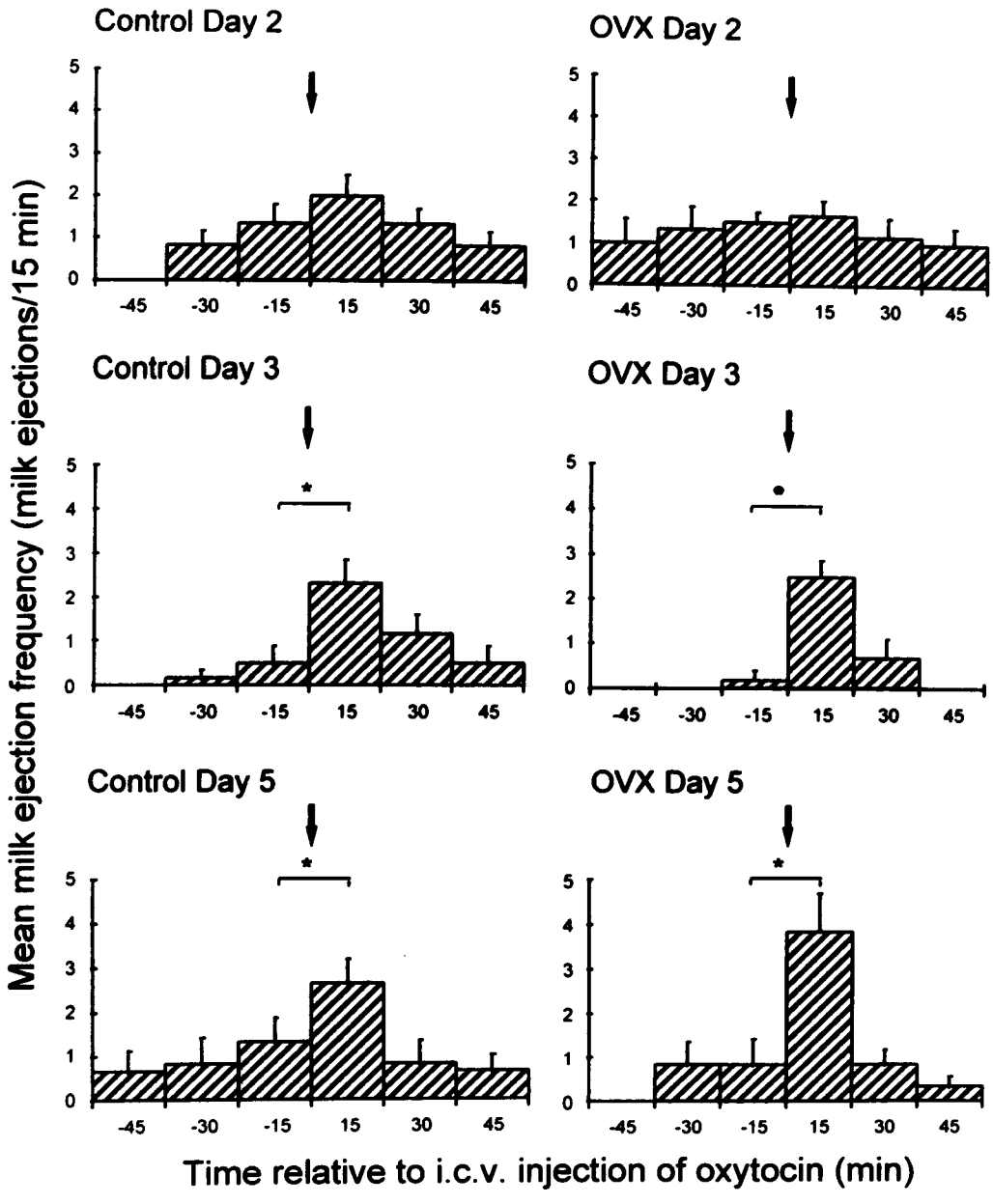

Fig. 7. Establishment of the facilitatory effect of central OT on the milk-ejection reflex on day 3 post-partum does not depend upon post-partum oestrus. Effect of i.c.v. injection of 2.2 ng of OT (arrow) on the milk-ejection reflex of anaesthetized suckled rats which had been subjected to sham surgery (control) or ovariectomy (OVX) on day 21 of pregnancy. Suckling tests were done at different days post-partum as indicated ($n = 6$ per group) and histograms show the number of milk-ejections (mean ± SEM) occurring in 15 min intervals centred around the i.c.v. injection of OT (*$P < 0.05$, Wilcoxon sign rank test).

h) of OT (Windle et al., 1997a). It has yet to be confirmed that this anxiolytic effect operates through the BNST, but analysis of changes in stress-induced activation of immediate early gene expression (da Costa et al., 1996) would indicate that this is the case. It is tempting, therefore, to suggest that this limbic region may have a pivotol role in co-ordinating the OT-induced suppression of stress which might otherwise interfere with neuroendocrine responses such as the milk-ejection reflex.

Why should the action of OT in the BNST be so dependent upon ovarian steroids? The anxiolytic effect of central OT to allow appropriate neuroendocrine (milk ejection) and behavioural (maternal behaviour) responses which might otherwise be prevented will only be advantageous at certain reproductive stages, such as following parturition. Under other situations, OT-induced anxiolysis would needlessly expose the individual to danger without any compensating benefit in improving reproductive functions. The dependency on ovarian steroids, particularly oestradiol, for OT to exert its central actions, may thus ensure that it is only at the most appropriate time in its life cycle that the individual has a reduced level of anxiety to enable expression of all the functions necessary for successful reproduction.

Acknowledgements

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SECTION 4

Vasopressin and autonomic functions

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CHAPTER 4.1

Role of brain vasopressin in regulation of blood pressure

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Using recent advances in brain physiological, neurohistochemical, and molecular biological techniques, it could be demonstrated that the central action of vasopressin (VP) is important in cardiovascular regulation and in the pathogenesis of hypertension. VP is now known to be located in the area of the brain involved in cardiovascular regulation. Furthermore, in various pathophysiological states, brain VP secretion is regulated separately from the peripheral VP secretion system. The role of brain VP in the regulation of the circadian rhythm of blood pressure is becoming a topic of major interest.

Introduction

Peripherally secreted vasopressin (VP) plays an important role in maintaining blood pressure in dehydration and hemorrhage, and contributes to the development and maintenance of some form of hypertension (Cowley and Liard, 1987). In contrast, the role of brain VP in blood pressure-regulation in pathophysiological states is not fully understood. Hemorrhage has been found to induce an elevation of the concentration of VP in cerebrospinal fluid in dogs (Wang et al., 1981), but whether or not brain VP contributes to maintaining blood pressure in hemorrhage is not clear. Similarly, DOC-salt hypertension leads to up-regulation of brain VP receptors (Swords et al., 1991), but it is unclear whether brain VP itself is essential in this type of hypertension. Furthermore, in various pathophysiological states, central administration of VP has either a hypertensive or hypotensive effect, depending on site and dose (Nashold et al., 1961; Matsuguchi et al., 1980, 1982; Riphagen and Pittman, 1980; Pittman et al., 1982; Feuerstein et al., 1983; Berecek et al., 1984a,b; Vallejo et al., 1984; Unger et al., 1984; Zerbe and Feuerstein, 1985; Porter and Brody, 1986; Tan and Tsou, 1986; Brattström et al., 1988; Imai et al., 1990; Gomez et al., 1993; Tuchihashi et al., 1993; Toba et al., 1994a; Liang et al., 1997; Smith and Ferguson, 1997). Thus, the basic physiology of the central vasopressinergic system in pathophysiological states such as dehydration, hemorrhage and hypertension is not yet fully understood.

VP is synthesized in the magnocellular neurons of the supraoptic (SON), paraventricular (PVN) and suprachiasmatic (SCN) nuclei of the hypothalamus. These nuclei send vasopressinergic fibers to the locus coeruleus, nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus and intermediolateral column of the spinal cord (Saper et al., 1976; Swanson, 1976; Buijs et al., 1978; Weindl and Sofroniew, 1980; Weindl and Sofroniew, 1985). These areas are important in the regulation of blood pressure and heart rate (Dorsa et al., 1983; Pearlmutter et al., 1983; Phillips et al., 1988). VP may modulate neural activity in these areas. We have demonstrated by microdialysis that VP is secreted, not only into the bloodstream, but also into the interstitial fluid of the brain in response to cholinergic stimulation, hemorrhage and osmotic stimuli (Ota et al., 1992, 1994a,b).

In addition, VP has been identified in nerve term-

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inals, and there is evidence for the synaptic release of VP in target areas (Buijs and Swaab, 1979; Buijs and van Heerikhuize, 1982; Pittman et al., 1982; Hawthorn et al., 1984). In fact, intracerebroventricular (i.c.v.) injection (Pittman et al., 1982; Berecek et al., 1984a; Unger et al., 1984; Zerbe and Feuerstein, 1985), intrathecal injection (Riphagen and Pittman, 1980, 1985; Porter and Brody, 1986; Tan and Tsou, 1986) or microinjection of VP into areas of the brain (Matsuguchi et al., 1982; Berecek et al., 1984b; Vallejo et al., 1984; Feuerstein, 1985) produces increases in blood pressure and heart rate. These cardiovascular changes are significantly attenuated by ganglion blockade, and α-adrenergic and β-adrenergic receptor antagonists (Riphagen and Pittman, 1980; Matsuguchi et al., 1982; Berecek et al., 1984a). These results suggest that the central effect of VP on cardiovascular function may be involved in regulation of the sympathetic outflow.

In this chapter, we discuss recent advances in research on brain VP and its effect on blood pressure.

Brain vasopressin and the regulation of blood pressure in the normal physiological state

Homozygous Brattleboro rats (diabetes insipidus rat), which have a hereditary lack of VP have a blood pressure higher than that in Long–Evans rats (Imai et al., 1990). Centrally administered VP V_1 or V_2 antagonists have no effect on blood pressure in Long–Evans rats (Imai et al., 1990; Toba et al., 1997) or in Wistar Kyoto rats (Budzikowski et al., 1996). Furthermore, chronic administration of low-dose VP (1 ng/h for 6 weeks) to homozygous Brattleboro rats fails to elevate blood pressure (Liang et al., 1997). In addition, continuous administration of very-low-dose VP (0.12 ng/kg per h, for 21 h) causes long-lasting hypotension after an initial hypertension in homozygous Brattleboro rats (Imai et al., 1990). In anesthetized rats, microinjection of a small dose of VP into the nucleus tractus solitarus (NTS) (Brattström et al., 1988) or the subfornical organ (Smith and Ferguson, 1997) decreases blood pressure. On the contrary, microinjection of VP into the nucleus reticularis lateralis or NTS also increased blood pressure in anesthetized rats (Matsuguchi et al., 1982; Gomez et al., 1993). Systolic blood pressure was slightly increased (7–12 mmHg) in Wistar rats treated with chronic low-dose VP (1 ng/h for 7 days) into the lateral cerebral ventricle (Tuchihashi et al., 1993).

These discrepant observations suggest that brain VP is involved in the action of both stimulatory and inhibitory neurons connecting to the cardiovascular regulatory center, and that the cardiovascular response to centrally applied VP differs according to dose, species and site of administration. The responses are strongly affected by anesthesia and the duration of VP administration. Overall, it has not yet been confirmed whether brain VP is important in blood pressure regulation in the normal physiological state.

Central vasopressin and its neural mechanism in regulation of blood pressure

The pressor response to exogenous VP in homozygous Brattleboro rats revealed that the response is not due to activation of endogenous brain or pituitary VP stores (Nussey et al., 1984). Williams and Johnson (1986) reported that the activity of the sympathetic nervous system is increased in Brattleboro rats, and hypothesized that this is mediated by the deficiency of VP in the central nervous system in this strain. In addition, Imai et al. (1990) reported that exogenous VP acts centrally as a cardiovascular inhibitor and stabilizer in Brattleboro rats.

A further experiment examining how VP exerts central cardiovascular control suggested that the responses depend primarily on alterations of autonomic efferent nerves from the central nervous system to both heart and vasculature. Schmid and Sharabi (1983) have shown that autonomic ganglion blockade with chlorisondamine can prevent the heart rate and blood pressure responses to i.c.v. administration of VP.

Furthermore, microinjection of VP directly into the NTS, where a VP action has been confirmed, alters the autonomic outflow to the cardiovascular system as a result of a central VP receptor-dependent mechanism (Matsuguchi et al., 1982). The site of action of VP may not be restricted to the NTS. Berecek et al. (1984b) reported that microinjection of VP into the locus coeruleus of conscious rats produces a consistent pressor response accompa-

nied by tachycardia. This response is blocked by peripheral administration of phentolamine. Gomez et al. (1993) reported that microinjection of VP into the nucleus reticulalis rostroventrolateralis (nRVL), which contains sympathoexcitatory neurons, produces a large dose-related increase in blood pressure. The nRVL was shown to be responsible for the mediation of signals from baro- and chemoreceptors (Reis et al., 1988).

There is some direct evidence for the release of VP in the brain. Pow and Morris (1989) using microdialysis showed that neurosecretory granules were released by exocytosis from the dendrites of the magnocellular neurons of the PVN. We also have reported the release of VP into the interstitial fluid of the brain (Ota et al., 1992, 1994a,b). In these studies with conscious freely moving rats, the microdialysis probe was placed adjacent to the PVN and was perfused with Ringer's solution. Dialysate-VP was increased in response to cholinergic stimulation, hemorrhage and hypertonic saline infusion. The concentration of VP released in the brain was much higher than the plasma VP concentration. This supports the assumption that central VP is insulated from circulating VP. Dialysate VP may reflect the neural activity of VPergic neurons. The synaptic release of VP may affect neural firing rate in several brain regions and this, together with the observation of VP binding in the same regions, suggests strongly that VP may act as a neurotransmitter in these regions (Dorsa et al., 1983; Pearlmutter et al., 1983; Phillips et al., 1988).

Recently, Funew et al. reported that microinjection of V_1 antagonist into the PVN immediately resulted in an increase in blood pressure and in plasma VP concentration in conscious rat (Faseb J. abstract). This finding suggests that central VP released around the PVN may act in a negative feedback loop to attenuate the increase in blood pressure.

VP may also act on baroreflex control directly from within the central nervous system. Cowley et al. (1980) reported that, in baroreceptor-denervated dogs, the pressor response to VP was augmented more than the response to norepinephrine. These findings were confirmed by Montani et al. (1980) and Liard et al. (1981). Izdebska et al. (1982) reported that i.c.v. injection of VP enhanced baroreflex gain. Imaizumi and Thames (1984) also reported that centrally administered VP enhances baroreflex function. These reports suggest that VP may act centrally to enhance the function of the baroreceptor reflex.

Brain vasopressin and regulation of blood pressure in pathophysiological states

Dehydration

Central vasopressin secretion in water-deprived animals

In earlier studies, the cerebrospinal fluid (CSF) concentration of VP was not changed in 24-h water-deprived conscious cats (Coleman and Reppert, 1985) and in 24-h water-deprived anesthetized rats (Mens et al., 1980), while in anesthetized steers, VP in CSF was elevated to 16 pg/ml after 96-h water deprivation (Doris and Bell, 1984). Recently we reported that increased plasma osmolality stimulates peripheral and central VP release in conscious rats (Ota et al., 1994a). Thus, VP secretion into the CSF in response to water deprivation appears to differ between species and/or magnitude of osmotic stimuli. To clarify this point, we measured the plasma and CSF concentration of VP in response to 24- and 48-h water deprivation in conscious rats (Toba et al., 1994a). The plasma concentration of VP was significantly elevated in water-deprived rats and the increase in VP was dependent on the duration of water deprivation. On the other hand, the concentration of VP in the microdialysis perfusate of the lateral ventricle did not differ between euhydrated control, 24- and 48-h water-deprived rats. In addition, in anesthetized euhydrated Long–Evans rats, the CSF concentration of VP was elevated to over 5 pg/ml (Liang et al., 1997). Together, these findings make it likely that in conscious animals, central VP is regulated separately from VP secretion in the periphery.

Role of central VP to modulate cardiovascular function in water-deprived animals

Because there is little information about this issue, we investigated the role of brain VP in the maintenance of blood pressure, using i.c.v. administration of a VP V_1 antagonist, OPC21268 (1-(1-

(4-(3-acetylaminoproxyl) benzoyl)-4-piperidyl)-3,4-dihydro-2(1H)-quinolinone), in conscious water-deprived rats (Toba et al., 1994b). Mean arterial blood pressure and heart rate were significantly decreased ($P < 0.05$–0.01) after injection of 30 and 300 μg/kg BW OPC21268. These changes were greater in the 48-h than in the 24-h water-deprived group. In the euhydrated rats, OPC21268 had no effect.

In an earlier study, i.c.v. injection of a V_1 antagonist, [1-(β-mercapto-β,β-cyclopentamethylene propionic acid-2-(*O*-methyl) tyrosine]arginine VP (TMe-VP), failed to alter mean arterial blood pressure or heart rate in 48-h water-deprived rats (Rochhold et al., 1984). The discrepancy among these findings may result from the dose or from chemical properties of the V_1 antagonist. The exact dose of V_1 antagonist required to block VP in vasopressinergic neurons is not known, and different results may be obtained if a higher dose is employed. In the present experiment, a non-peptide VP V_1 antagonist (OPC21268) was used. It is possible that this compound is distributed in the brain differently from TMe-VP.

Since the CSF VP concentration in water-deprived rats was not elevated (see above), and central VP was demonstrated to enhance sympathetic nerve activity (Schmid and Sharabi, 1983; Unger et al., 1984), upregulation of the VP V_1 receptor in the brain is suggested to be involved in the mechanisms of the hypotensive and bradycardiac effects of OPC21268 in water-deprived rats.

Hemorrhage

Central vasopressin secretion in hemorrhage

In severe hemorrhage in dogs, CSF concentration of VP exceeds 400 pg/ml (Wang et al., 1981). The source of brain VP in hemorrhage has remained unclear, because the possibility of leakage of VP through the blood–brain barrier at an extremely high concentration was not examined. We demonstrated that brain VP was increased in the microdialysis perfusate of the paraventricular nucleus in conscious rats with hemorrhage (Ota et al., 1994a). This result implies that a major part of the increase in VP results from enhanced secretion of VP into the brain.

Role of central VP to modulate cardio-vascular function in hemorrhage

The contribution of central VP to maintain blood pressure in hemorrhage remains controversial. Bolus injection of a VP V_1 receptor antagonist, [d(CH_2)$_5$Tyr(Me)]VP, into the lateral cerebral ventricle aggravated hypotension in rats (Johnson et al., 1988). In addition, microinjection of [d(CH_2)$_5$Tyr(Me)]VP into the bilateral nucleus reticulalis rostroventrolateralis (nRVL) decreased blood pressure strongly from 110 to 50 mmHg (Gomez et al., 1993). On the contrary, continuous infusion of the VP V_1 receptor antagonist for 60 min into the lateral cerebral ventricle resulted in an initial attenuation of the hypotension due to hemorrhage (Budzikowski et al., 1996). These findings suggest that brain VP in the nRVL acts to maintain blood pressure in hemorrhage through enhancement of the baroreflex, while VP has an opposite effect in the brain tissue around the lateral ventricle. However, because the time course of the CSF or tissue concentration of VP was not examined in these studies, and because a delayed increase in CSF VP was shown in hemorrhage (Wang et al., 1985), further studies are warranted to examine the role of central VP in the maintenance of blood pressure in the late stage of hemorrhage.

Other pathophysiological conditions

Experimental hypoxia, hypercapnia and metabolic acidosis were all found by Wang et al. (1984) to be stimuli that increase plasma VP concentration. Severe hypoxia and hypercapnia cause an increase of CSF VP concentration, while metabolic acidosis achieved by the infusion of HCl into the lateral cerebral ventricle has no effect. These results confirmed that central and peripheral excretion of VP are regulated separately, although it remains unclear whether these elevations of CSF VP regulate the cardiovascular response in each pathological state.

It has been shown that the pressor response to exogenous VP (Crofton et al., 1988; Toba et al., 1991), VP secretion in response to hemorrhage and osmotic stimuli (Crofton and Share, 1990; Ota et al., 1994a,b), and the development of

DOCA-salt hypertension (Ouchi et al., 1987, 1988) are different in male and female rats. These sexually dimorphic responses may be related to the interaction of VP and sex steroids in the brain, since brain-stem catecholamine neurons are target sites for sex steroid hormones (Heritage et al., 1980). Indeed, we demonstrated that VP release in response to osmotic stimuli both into brain tissue and to the periphery, was greater in female rats, although the changes in blood pressure were similar (Ota et al., 1994a), and that the pressor response to ICV VP was greater in males than in females (Toba et al., 1994a). Together, these findings suggest that the attenuated pressor response to either peripheral and central VP in the female is compensated for by enhanced peripheral and central secretion of VP.

Brain VP and hypertension

Role of brain VP in DOCA-salt hypertension

It has been demonstrated that arginine VP is important for the development and maintenance of DOCA-salt hypertension (Friedman et al., 1960; Möhring et al., 1977, 1979; Crofton et al., 1979b; Berecek et al., 1982a). DOCA-salt hypertension fails to develop in hereditary diabetes insipidus (DI) rats, while DOCA-salt hypertension is evoked by peripherally administered VP in DI rats (Berecek et al., 1982b; Berecek and Brody, 1982; Saito and Yajima, 1982). However, several studies involving the peripheral administration of VP antagonists showed that the contribution of VP to resting blood pressure in DOCA-salt hypertension is slight (Rabito et al., 1981; Rascher et al., 1981; Mento et al., 1984). Furthermore, in DI rats with a lesion of the anteroventral region of the third ventricle, the development of hypertension was incomplete even if VP was given as replacement by peripheral administration (Berecek et al., 1982a). On the other hand, it has been shown that VP and neurogenic mechanisms are involved in DOCA-salt hypertension (Matsuguchi and Schmid, 1982a; Berecek et al., 1984a; Toba et al., 1994b). First, an intact innervation is required for maintenance of the vascular resistance augmented by VP in DOCA-salt hypertension (Matsuguchi and Schmid, 1982a), while the baroreceptor reflex is impaired in DOCA-salt hypertensive rats (Matsuguchi and Schmid, 1982b). Second, VP administered into the lateral cerebral ventricle produces a greater pressor response in DOCA-salt hypertensive rats than in normotensive rats (Berecek et al., 1984a). Third, an i.c.v. injected VP V_1 receptor antagonist was demonstrated to have a depressor and bradycardiac effect in DOCA-salt hypertensive rats (Toba et al., 1994b). Finally, the number of [^{3}H]arginine VP binding sites in the brain is increased in DOCA-salt hypertensive rats (Swords et al., 1991). These results indicate that VP V_1 receptors are up-regulated in DOCA-salt hypertension, and that interaction of VP and the central nervous system plays a pivotal role in DOCA-salt hypertension through induction of enhanced vascular reactivity or through a baroreflex function.

However, it is still unclear whether central VP is itself essential for the development of DOCA-salt hypertension. We evaluated the effect of chronic ICV replacement of VP on systolic blood pressure, heart rate and sodium-water metabolism in homozygous Brattleboro (DI) rats treated with DOCA-salt. Neither ICV infusion of VP nor DOCA-salt treatment alone had any effect on systolic blood pressure. On the other hand, hypertension was partially restored by treatment with DOCA-salt combined with ICV infusion of VP, though the magnitude of the increase in systolic blood pressure was less than a third of the increase in blood pressure in Long–Evans rats treated with an i.c.v. infusion of 0.9% NaCl and DOCA-salt. These hypertensive homozygous Brattleboro rats had an increase in fluid intake and urinary sodium excretion similar to that in DOCA-salt hypertensive Long–Evans rats.

On the basis of these results, it would appear that central VP is required for the complete development of DOCA-salt hypertension. The mechanism partly involves sodium intake enhanced through the additive effect of central VP and DOCA-salt.

Role of brain VP in spontaneously hypertensive rats (SHR)

Genetic models of hypertension such as the spontaneously hypertensive rat (SHR), Dahl salt-sensitive rats, and the Sabra rat are characterized by an

increased sympathetic nerve activity or by alterations in brain catecholamines (De Champlain et al., 1976; Saavedra et al., 1978; Feuerstein et al., 1982). Sladek et al. (1986) reported that SHR show abnormalities in the distribution of central catecholaminergic and VPergic neurons in comparison to that in normotensive rats. Several reports suggest that there are high central and peripheral levels of VP and increased VP gene expression in PVN and SON (Crofton et al., 1978; Möhring et al., 1979; Sladek et al., 1988; van Tol et al., 1988). On the other hand, there are reports of decreased VP levels in the brain nucleus of SHR and of attenuation of hypertension in SHR after lesions of the PVN (Möhring et al., 1980; Rascher et al., 1982; Morris et al., 1983; Ciriello et al., 1984). Central VP may contribute to the development of hypertension at an earlier age in SHR (less than 4 weeks) (Berecek and Swords, 1990), since the VP content in the hypothalamic paraventricular nucleus of juvenile SHR is reduced after (more than 6 weeks) but not before (3 weeks) the development of hypertension (Morris et al., 1983).

Conversely, studies with the Sabra hypertensive rat showed a 3-fold elevation of VP in the NTS compared with the level in normotensive genetic controls (Feuerstein et al., 1982). These results all suggest that VP plays an important role in noradrenergic innervation of the brain, and that an increase in VP content in SHR might alter both catecholaminergic innervation, and the increase in sympathetic nerve activity that characterizes hypertension in this model. SHR are characterized by hyperreactivity of the sympathetic nervous system. VP may in the early stages of hypertension, alter the sympathetic outflow via an effect on central neural structures controlling the sympathetic nervous system.

Brain VP interaction with other centrally vasoactive substances

Nitric oxide

Nitric oxide (NO) has recently been recognized as a possible neuronal messenger, however, its physiological neuroregulatory mechanisms have not yet been fully studied. It now appears that NO may play a role in central regulation of blood pressure (Garthwaite and Boulton, 1995; Vincent, 1995). Microinjection of analogs of L-arginine, inhibiting NO synthesis, into the NTS and rostral ventrolateral medulla (RVLM), has been found to cause an increase of blood pressure and sympathetic nerve activity (Harada et al., 1993; Zanzinger et al., 1995). There are several reports that administration of NO donors into the PVN, NTS and RVLM results in a decrease in blood pressure and sympathetic nerve activity (Hom et al., 1994; Zanzinger et al., 1995). On the other hand, we found that i.c.v. administration of the NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), or the NO biosynthetic precursor, L-arginine, increased blood pressure and VP release (Ota et al., 1993). In addition, some evidence suggests the existence of an interaction between brain NO and VP. Sanchez et al. (1994) have reported that NADPH-diaphorase co-exists with VP in hypothalamic magnocellular neurons. Moreover, the presence of NO synthase has been demonstrated in several brain areas, particularly the PVN and SON (Bredt et al., 1990; Snyder and Bredt, 1991). A high density of NO synthase is also found in the nerve terminals of the posterior pituitary (Bredt et al., 1990). These findings suggest that NO may play some role in the regulation of cardiovascular function and VP release. Recently, Paczwa et al. (1997) reported that centrally released VP increases the availability of NO in the brain cardiovascular regions, where NO plays a compensatory role by reducing the pressor effect of VP. This compensatory mechanism is enhanced in SHR. Together, these reports indicate that NO produced in the central nervous system attenuates the central pressor effect of VP.

Angiotensin II

The renin-angiotensin system has been found not only in tissues such as the heart, kidney and vasculature, but also in the brain. The brain renin-angiotensin system appears to be regulated independently from the circulating renin-angiotensin system (Lind et al., 1985a; Phillips, 1987; Unger et al., 1988; Whiting et al., 1991). Sladek and Joynt (1979) reported that angiotensin II (AII) stimulates

VP release from the rat hypothalamo-neurohypophyseal system.

Receptors for AII have been identified immunohistochemically in the rat subfornical organ (SFO) (Lind et al., 1985b). The action of AII on the SFO has been studied extensively, and it is clear that AII acts on receptors in the SFO to stimulate neural pathways subserving water drinking and pressor responses in the rat (Simpson et al., 1978; Simpson, 1981). These results suggest that AII may stimulate VP release through AII receptor stimulation in the SFO. In addition, Crofton et al. (1979a) reported that an angiotensin converting enzyme (ACE) inhibitor decreases the urinary excretion of VP, suggesting suppression of VP release. These data raise the possibility that modulation of VP synthesis and secretion may be important functions of the central renin-angiotensin system. In fact, ACE, AII and renin were found to be highly concentrated in the PVN and SON, which are the VP-synthesizing nuclei in the hypothalamus (Lind et al., 1985a).

Hogarty et al. (1992) reported that AII given centrally produces an increase in blood pressure and in the motivation to drink. The physiological mechanisms that mediate the pressor response include release of VP and activation of the sympathetic nervous system. The authors demonstrated that the pressor response to AII is predominantly AT1-mediated, while the VP response may be mediated by both AT1 and AT2 receptors. Moreover, microdialysis experiments showed VP release in the PVN and SON to be mediated by the AII-stimulated release of norepinephrine and activation of α1-receptors in these nuclei (Qadri et al., 1993). These results suggest that the central cardiovascular effects of VP are modulated by the central renin-angiotensin system.

Future aspects

The suprachiasmatic nucleus (SCN) of the hypothalamus appears to function as a circadian clock. Although a variety of neuropeptides have been localized within SCN neurons, little is known about their role in circadian timekeeping (Moore, 1983; Moore and Card, 1985; Van den Pol and Tsujimoto, 1985). Swaab et al. (1975) and Vandesande et al. (1975) reported that the SCN has a prominent population of VP-containing neurons. A potential candidate for driving this circadian rhythm is VP, the only transmitter thus far identified to have an intrinsic rhythm of mRNA transcription (Uhl and Reppert, 1986; Burbach et al., 1988; Young et al., 1993).

There are several reports of a circadian rhythm of VP and VP mRNA in the SCN (Noto et al., 1983; Murakami et al., 1991; Yamase et al., 1991; Watanabe et al., 1993; Cagampang et al., 1994). In addition, efferent projections of the SCN to the median eminence, periventricular region, subparaventricular region ventral to the PVN, dorsomedial hypothalamus (DMH) and PVN have been found (Swansen and Cowan, 1975; Buijs, 1978; Sofroniew and Weindl, 1978; Kucera and Favrod, 1979; Berk and Finkelstein, 1981; Stephan et al., 1981; Hoorneman and Buijs, 1982; Watts and Swansen, 1987; Watts et al., 1987). These areas are important in the regulation of sympathetic and parasympathetic nerve activity. In fact, Halberg et al. (1965) reported that heart rate rhythms run according to the original biological clock in men under dark, silent and isolated conditions. These data suggest that blood pressure rhythms are at least partially under the control of the SCN (Halberg et al., 1965). Sano et al. (1995) demonstrated that the SCN is important not only for generating the circadian rhythm of blood pressure, heart rate and locomotor activity, but also for buffering the short-term variability of blood pressure in rats (Sano et al., 1995). Moreover, Janssen et al. (1994) reported that the circadian rhythm for blood pressure and heart rate is abolished in SCN-lesioned rats. These findings indicate that long-term, but not short-term blood pressure and heart rate variability is largely determined by SCN-controlled activity levels.

Thus, another important role of VP in the brain may be regulation of the circadian rhythm of the SCN. VP is an important neurotransmitter in the regulation of sympathetic and parasympathetic nerve activity in the PVN, ventrolateral hypothalamus (VMH) and lateral hypothalamus, which is the center for regulation of autonomic nerve function. The interaction between VP in the SCN and the blood pressure rhythm is a promising topic for further study.

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CHAPTER 4.2

Vasopressin neurotransmission and the control of circadian rhythms in the suprachiasmatic nucleus

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Vasopressin (VP) is one of the principal transmitters in the suprachiasmatic nucleus (SCN). Approximately 20% of neurones in the dorsomedial division of the SCN synthesize the peptide and a high proportion of SCN neurones (>40%) are excited by VP acting through the V_1 receptor. This suggests that VP may act as a feedback regulator of electrical activity within the nucleus. Such an intrinsic excitatory signal can be demonstrated by perifusion with a V_1 antagonist which reduces spontaneous neural activity. As the synthesis and release of VP occurs in a circadian manner, this leads to a variable feedback excitation which may contribute to the circadian pattern of activity of the neural clock. This role in amplifying rhythmicity is supported by observations that animals deficient in VP show a reduced circadian amplitude of behavioural rhythms (e.g. locomotor and cortical electroencephalographic rhythms). VP expression declines during ageing and although aged animals show no change in the proportion of SCN neurones excited by VP, the rhythm of spontaneous electrical activity shows a progressive decline, consistent with the reduced endogenous excitatory feedback. However, the homozygous Brattleboro rat which lacks any VP expression still maintains rhythms of electrical activity, indicating that VP is not the sole factor generating circadian activity. The generation of this rhythmicity may depend upon the interaction of VP with other transmitter systems, such as the inhibitory transmitters somatostatin and GABA which show a circadian variation in efficacy. In addition to its role in feedback amplification of the endogenous rhythm of electrical activity, VP also functions as part of the efferent signal to the rest of the CNS where it potentially regulates a number of behavioural and physiological rhythms, including the circadian activity of the hypothalamo–pituitary–adrenal axis. Thus, the combined amplification and signalling functions makes VP an important component of the neuronal clock function in mammals.

Introduction

In mammals, circadian rhythms are dependent upon signals generated within the hypothalamic suprachiasmatic nuclei (SCN) and, as such, the SCN is considered as the principal neural clock. There are several features of the endogenous clock mechanism which are important for the generation of normal circadian rhythms, and these include: (i) the generation of an approximately 24 h rhythm; (ii) the amplification of this rhythm (i.e. increasing the difference between nadir and acrophase) to produce a biologically significant signal; (iii) the control of the relationship between internal and external (environmental) rhythms (i.e. entrainment); and (iv) the transmission of the rhythm to other parts of the brain. Each of these features may be regulated by separate neural circuits within the SCN. Expression of the activity of the clock may be measured by several indices including: the behaviour (e.g. locomotion, feeding, drinking) or physiology (e.g. body temperature) of the intact animal; the expression of both cell regulatory genes or neurotransmitter-related genes and their particular products; or, more directly, the electrical activity of the neurones themselves. In respect of the latter, neurones of the SCN display a circadian

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cycle of electrical activity, which is intrinsic to the nucleus and persists after isolation in vitro (Green and Gillette, 1982; Groos and Hendricks, 1982; Shibata et al., 1982, 1984; Gillette and Reppert, 1987; Bos and Mirmiran, 1990). This electrical activity peaks during the subjective light phase and appears to depend upon excitatory synaptic activity, since recordings made under conditions of synaptic blockade display a constant level of activity similar to that during the night time nadir (Shibata et al., 1984; Mirmiran et al., 1995). However, the neurochemical signals regulating this rhythm are poorly understood. [Arg^8]vasopressin (VP) is one of the principal endogenous transmitters synthesized within the SCN and there are several lines of evidence to suggest that VP may participate in the generation of circadian rhythms: (i) Both the expression of VP mRNA (Burbach et al., 1988; Carter and Murphy, 1989; Cagampang et al., 1994) and the content of VP peptide (Tominaga et al., 1992) within the SCN show circadian rhythms which are independent of extrinsic cues. Indeed, VP is the only transmitter found within the SCN which has been shown to have a persistent rhythm after removal from the animal (Inouye and Shibata, 1994); (ii) The expression of VP V_1 receptor mRNA (Ostrowski et al., 1992) and the cell surface expression of V_1 receptors (Kremarik et al., 1995) show circadian rhythms which have a phase relationship with VP peptide release but do not depend upon the peptide (Young et al., 1993); (iii) The secretion of VP from the SCN measured both in vivo (Reppert et al., 1981; Schwartz and Reppert, 1985; Kalsbeek et al., 1995) and in vitro (Earnest and Sladek, 1987; Gillette and Reppert, 1987; Earnest et al., 1991; Murakami et al., 1991; Watanabe et al., 1993; Shinohara et al., 1994; Tominaga et al., 1994) show circadian cycles which again have a phase relationship with VP synthesis. The regulation of VP expression in the SCN has been reviewed (Inouye and Shibata, 1994) as has its role in circadian time keeping (Reghunandanan et al., 1991, 1993). In this chapter we will describe the role of VP in SCN neurotransmission and the evidence that VP may be involved in both the amplitude regulation and output features of the circadian rhythm generator.

Circadian patterns of electrical activity in the SCN

SCN neurones exhibit a circadian pattern of spontaneous electrical activity in vivo (Meijer and Rietveld, 1989) which persists after in vitro isolation in brain tissue slices (Green and Gillette, 1982; Groos and Hendricks, 1982; Shibata et al., 1982, 1984; Gillette and Reppert, 1987; Ingram et al., 1996) and in either organotypic (Bos and Mirmiran, 1990; Aronson et al., 1993; Mirmiran et al., 1995) or dissociated cell cultures (Welsh et al., 1995). Recordings from long-term organotypic (Mirmiran et al., 1995) and dispersed cell cultures (Welsh et al., 1995) show that some but not all SCN neurones display circadian patterns of electrical activity. Importantly, although synaptic activity appears to be necessary to maintain fully synchronized circadian rhythmicity, suggesting a role for the endogenous connectivity within the SCN (Mirmiran et al., 1995), evidence from long duration application of tetrodotoxin to cultured SCN neurones demonstrate that the persistence of the underlying circadian oscillator does not depend upon synaptic input (Welsh et al., 1995). All our work described in this chapter has been carried out on brain slices since this provides a convenient model for isolating the SCN neurones, while maintaining the intrinsic connectivity of the various cell types. Using this model we have focused on the role of VP as this is the principal peptide displaying circadian expression.

Circadian pattern of synthesis and secretion of VP in the SCN

VP is the most abundant peptide found in the SCN, and is expressed within parvocellular neurones in the dorsomedial (DM) region of the SCN where approximately 20% of neurones are immunoreactive for VP (Sofroniew and Weindl, 1980). Expression of VP shows an endogenous circadian rhythm, involving changes in mRNA levels (Uhl and Reppert, 1986; Burbach et al., 1988; Carter and Murphy, 1989; Cagampang et al., 1994) which seem to be regulated within the nucleus by attenuation of transcriptional activity (Carter and Murphy, 1992). In addition there is a

diurnal variation in molecular size due to differential polyadenylation, with the longer and more stable 740 nucleotide mRNA expressed during the light phase (Robinson et al., 1988; Carter and Murphy, 1989). Correlated with and dependent upon these cycles of transcription and polyadenylation is a rhythm of VP release which is maintained in vitro and peaks during the subjective light phase (Earnest and Sladek, 1987; Gillette and Reppert, 1987; Earnest et al., 1991; Murakami et al., 1991; Watanabe et al., 1993; Shinohara et al., 1994; Tominaga et al., 1994). The remarkable correlation between the in vitro cycles of VP release and of electrical activity (both peaking in the middle of the light phase), has led to the hypothesis that the endogenous cycle of VP release contributes to the cycle of electrical activity and, thereby, functions as integral part of the circadian clock mechanism. However, a causal link between these two rhythms has been previously dismissed as the peak of electrical activity occurs slightly out of phase with the peak of peptide secretion, peptide content or VP mRNA levels. However, this assumes that no other factors are playing a part in the response, such as cyclical changes in receptor expression or interactions with inhibitory transmitters. In the following sections these possibilities will be considered.

Role of VP in intranuclear neurotransmission and circadian patterns of activity

There is considerable evidence that VP synthesized within the SCN is involved in intranuclear circuits, which have the potential to participate in the generation and integration of circadian signals. Immunoelectronmicroscopy has shown the presence of VP-immunoreactive synapses which contact a number of neurochemically different cell types (van den Pol and Gorcs, 1986; Castel et al., 1990; Daikoku et al., 1992). VP synapses have been seen on VP neurones and may participate either in the synchronization of the population of VP-producing neurones, or in recurrent control that may be important for the resetting of the clock. Local VP circuits exist within the SCN involving soma-somatic appositions of VP neurones and an extensive axo-dendritic network innervating neurones in both the ipsilateral and contralateral SCN (Castel et al., 1990). Although VP fibres appear to be concentrated in the DM division of the SCN (Daikoku et al., 1992), V_1 receptors are equally distributed throughout the nucleus (Kremarik et al., 1995). This is in agreement with our observation that there is no regional localisation of VP responsive neurones (unpublished observations), although others have reported that a greater proportion of responsive neurones are found in the DM division of the hamster SCN (Liou and Albers, 1989).

Both our own studies in rat (Mihai et al., 1994a,b; Ingram et al., 1996) and those of others in rat (Shibata and Moore, 1988) and hamster (Liou and Albers, 1989) have shown that a high proportion of SCN neurones (approximately 40%) show excitatory responses to VP in vitro (Fig. 1). In general, perfusion with doses of 10^{-7} M or greater are required in order to observe significant excitation, although lower doses may be effective during the subjective dark phase when endogenous peptide release is reduced. Indeed, both ourselves and others (Liou and Albers, 1989) have shown larger responses and a greater proportion of SCN neurones responding to VP during the dark phase of the circadian cycle. This difference may arise either from the circadian variation in VP receptor mRNA expression (Young et al., 1993), or from the fact that endogenous excitatory tone is reduced during the dark phase. The excitatory effects of VP can be blocked by co-administration of the receptor antagonists $d(CH_2)_5$[D-Tyr(OMe)^2,Val^4,Cit^8]VP (Fig. 1C; Mihai et al., 1994a) or $d(CH_2)_5$[Tyr(OMe)^2]VP (Liou and Albers, 1989), indicating a receptor-mediated effect. A much smaller population of SCN neurones (<5%) show inhibitory responses to VP (Mihai et al., 1994a) and the possibility that these effects may be mediated via inhibitory interneurones is indicated by the small increase in activity following application of a receptor antagonist (Mihai et al., 1994b). Such interneurones may include those synthesizing either somatostatin or GABA (see below).

Autoradiographic studies have shown the presence of binding sites for either [^{3}H]VP (Freund-Mercier et al., 1987, 1988; Tribollet et al., 1988) or the highly selective linear V_1 antago-

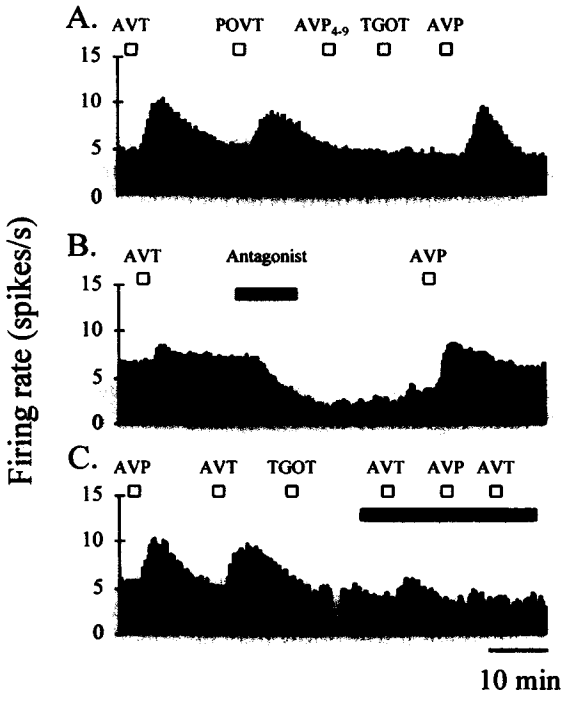

Fig. 1. Electrophysiological actions of VP and related peptides in the SCN. Neurones showing excitatory responses to VP, [Arg^8]vasotocin (AVT) and the V_{1a} agonist, [Phe^2,Orn^8]vasotocin (POVT) at 10^{-6} (A,B) or 10^{-7} M (C). These neurones showed no response to the OT agonist [Thr^4,Gly^7]oxytocin (TGOT) (A,C) or the VP metabolite [*p*Glu^4,Cyt^6]VP 4–9 (VP 4–9) (A). In a proportion of neurones application of the VP antagonist d(CH$_2$)$_5$[Tyr(OEt)^2,Val^4,Cit^8]VP (5×10^{-7} M) caused a marked suppression of basal activity, indicative of an endogenous excitatory drive in the tissue (B). This dose of antagonist was able to block the excitatory effects of exogenous VP and AVT (C).

nist Phaa,D-Tyr(Me),Phe,Gln,Asn,Arg,Pro,Arg,-Tyr-NH$_2$ (Kremarik et al., 1995), and V_{1a} (but not V_2) receptor mRNA transcripts has been detected in the SCN (Ostrowski et al., 1992; Young et al., 1993). Consistent with the presence of V_{1a} receptors, SCN neurones show excitatory responses to the V_{1a} selective agonist [Phe^2,Orn^8]vasotocin (Fig.1A; Liou and Albers, 1989, Mihai et al., 1994a), but not the V_2 agonist [Val^4,D-Arg^8]VP (Liou and Albers, 1989). Interestingly, despite the fact that the oxytocin receptor-selective agonist [Thr^4,Gly^7]oxytocin (TGOT) has no effect on most SCN neurones (e.g. Fig.1A,C), a small sub-population of neurones are excited (Mihai et al., 1994a), consistent with the presence of a low density of binding sites for the iodinated OT antagonist d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Orn^8,Tyr-NH$_2^9$]-vasotocin (Kremarik et al., 1995).

In addition to showing the excitatory effects of TGOT (Tolchard and Ingram, 1993) and VP, our studies in the brain-stem dorsal vagal complex have shown that the ancestral hybrid peptide [Arg^8]vasotocin (VT) can have excitatory effects, apparently independent of OT or VP receptors (Ingram and Tolchard, 1994). VP-responsive neurones in the SCN are also excited by VT (e.g. Fig. 1) and a few neurones show highly preferential responses to this peptide (Mihai et al., 1994a). This suggests that a receptor for VT may be expressed in several parts of the mammalian brain including the SCN. However, this receptor appears not to be that activated by the common metabolite [*p*Glu^4,Cyt^6]VP 4–9 as we have been unable to detect any effect of this peptide on SCN neurones (Fig. 1A; Mihai et al., 1994a).

Our recordings have provided two important pieces of evidence which suggest that VP may be contributing to the circadian cycle of electrical activity. Firstly, during the application of a V_1 antagonist the otherwise stable basal activity showed a reversible decline in activity, indicative of an endogenous excitatory tone (Fig. 1B; Mihai et al., 1994a). Interestingly, the magnitude of this effect was greater during the light phase when endogenous VP secretion is highest than during the dark phase (Mihai et al., 1994b). Secondly, the circadian variation in tonic basal activity is much greater for those neurones which are responsive to VP than for those which are insensitive. This is consistent with the observations of both circadian and non-circadian neurones in culture (Mirmiran et al., 1995; Welsh et al., 1995), and suggests that the ability to respond to VP (i.e., expression of VP receptors) may be a marker for this sub-population of highly circadian neurones. However, we currently have no evidence for the identity of these neurones.

The ability of VP to interact with rhythm generating mechanisms is supported by in vivo beha-

vioural data. Injection of an VP antagonist into the SCN causes a shift in the ratio of water and food intake between light and dark phases of the cycle, without abolishing the circadian difference (Reghunandanan et al., 1987, 1992). Similarly, continuous i.c.v. infusion of anti-VP antiserum or a V_1 receptor antagonist significantly reduces the power of the locomotor rhythm, reducing the active time and increasing slow-wave sleep (Arnauld et al., 1989), while the power of the rhythm is increased by VP (Kruisbrink et al., 1987). Thus, while not essential for rhythm generation, these data suggest that VP may be important for controlling the amplitude of circadian rhythms.

Circadian rhythms in the absence of endogenous VP

Some of the best evidence for the role(s) of VP in the chronobiology of the SCN has come from examination of models of altered peptide expression. Unfortunately, no transgenic animal bearing the VP gene has been shown to express VP in the SCN (Zeng et al., 1994, Waller et al., 1996), and there is no VP-knockout rat with which to study changes in rhythmicity. However, there are several natural states of absence of VP, including the homozygous Brattleboro rat and the mink.

The Brattleboro strain of Long–Evans rat carries a single base deletion in Exon B of the VP gene. In the homozygous animal, this recessive mutation results in an inability to process the translated gene product to VP, and immunocytochemical staining has shown the complete absence of VP in the SCN (van Leeuwen et al., 1989). Nevertheless, in spite of this deficiency, homozygous Brattleboro rats continue to display circadian rhythms of cortical EEG (Brown and Nunez, 1989), motor activity (Peterson et al., 1980; Groblewski et al., 1981), drinking behaviour (Peterson et al., 1980; Stoinev and Ikonomov, 1990) and pineal *N*-acetyltransferase levels (Peterson et al., 1980; Schröder et al., 1988). Furthermore, the rhythms of expression of VP and V_{1a} receptor genes in homozygous animals are identical with controls, despite the absence of any translated VP which may feedback on the pacemaker (Uhl and Reppert, 1986; Young et al., 1993). These observations have led to the conclusion that, despite its marked circadian expression, VP has no role in the generation of circadian rhythms by the SCN.

We have studied VP effects in the SCN of Brattleboro rats in order to determine any possible role of VP in controlling circadian rhythms (Ingram et al., 1996). The density of [^{3}H]VP binding sites is much greater in the homozygous Brattleboro rat than in rats expressing VP (Freund-Mercier et al., 1988; Snijdewint et al., 1989), and this was reflected by a significantly greater proportion of neurones responding to VP, and in the observation of responses to lower doses of VP than normally seen in Wistar rats. This difference in receptor expression appears not to be due to differences in transcription rates (Young et al., 1993) but may reflect the availability of free receptors or homologous receptor down-regulation which might occur in the presence of endogenous peptide. A similar proportion of neurones (4%) were inhibited by VP, suggesting that effects of VP mediated through inhibitory interneurones would also be affected by the absence of the peptide.

Compared to Wistar rats, mean basal activity of VP-responsive neurones was lower in the Brattleboro rats, consistent with the absence of a tonic excitatory drive. Furthermore, when the population of neurones was classified on the basis of the response to VP, the difference in spontaneous activity between the light and dark phases of the cycle was much greater for the responsive neurones. This suggests that, although the excitation by endogenous VP is not the principal mechanism driving the circadian rhythm of electrical activity, like in normal animals (Mihai et al., 1994a), VP sensitivity appears to be a marker for a sub-population of highly circadian SCN neurones. Finally, when we compared the spontaneous activity between heterozygous and homozygous rats, the peak activity during the subjective light phase was significantly lower in the homozygous animals, consistent with a lack of VP feedback at this time. Interestingly the absolute difference in firing rate in the light phase was very similar to the antagonist-induced decrease in firing rate seen in Wistar rats (Mihai et al., 1994b). Furthermore, in this study of the effects of the receptor antagonist (Mihai et al., 1994b) only half the SCN neurones showed a decrease in

spontaneous activity in response to the peptide. However, despite the apparent absence of any tonic excitation, the antagonist-insensitive neurones still showed a significant circadian variation in spontaneous activity suggesting that, like in the homozygous Brattleboro rat, VP was not the principal generator of the circadian rhythm. Thus, all these data point to the fact that VP is not essential for generation of circadian rhythms but serves to amplify the light-dark differences.

The effect of the absence of VP on the power of circadian rhythms of electrical activity are also reflected in the other behaviours by Brattleboro rats. Although homozygous animals maintain free-running rhythms of drinking and locomotor activity under constant low light (Peterson et al., 1980; Groblewski et al., 1981), the diurnal difference is much less marked compared to heterozygous controls. Furthermore, homozygous animals show a dampening of EEG rhythms with a greater proportion of total slow-wave and paradoxical sleep during the dark (normally active) period (Brown and Nunez, 1989), a difference which is maintained even after correcting their drinking by i.v. VP or water (Groblewski et al., 1981; Danguir, 1983).

Although SCN slices from homozygous Brattleboro rats show a significant increase in activity during the second day of incubation indicating a free-running rhythm (e.g. Fig. 2), the peak of activity is smaller and shorter than that in the first light phase (Ingram et al., 1996). Although it is not known what maintains the robust rhythms of VP synthesis and secretion which can be maintained over many cycles (Murakami et al., 1991), the ability of tetrodotoxin to permanently suppress the VP rhythm when administered specifically during the late subjective light phase (Earnest et al., 1991), suggests that sodium-dependent electrical activity is necessary at this time to re-set the clock for the next circadian cycle. Thus, the absence of VP-induced excitation during this period in Brattleboro rat slices may have accounted for the dampening of the activity rhythm. Therefore, we have examined whether increasing the feedback effects of VP at this time can affect activity during the subsequent cycle. Fig. 2 shows the pattern of activity in the SCN of homozygous Brattleboro rats on the second day in vitro with or without exposure to VP during the late light phase of the first day.

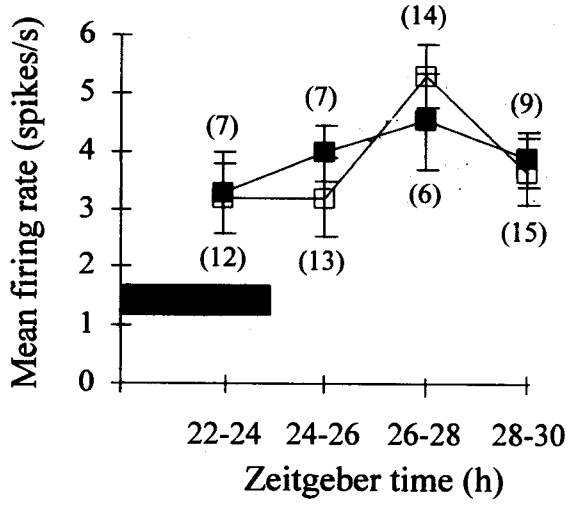

Fig. 2. Circadian rhythm of SCN neurone activity in VP-deficient Brattleboro rats. Tissue slices from homozygous animals were prepared at ZT 0100 h and during the late light phase (ZT 0600–1200 h) were exposed to 5×10^{-8}M VP (closed symbols) or left as controls (open symbols). The figure shows the mean ± SEM firing rate of neurones recorded during the light phase of the second circadian cycle. Numbers of neurones are shown in parentheses.

Although the data show that there is a significant increase during the light phase, there was no shift in the peak of the activity rhythm, suggesting that VP is not involved in re-setting of the clock. This is in agreement with previous reports that VP does not cause a phase shift of the neuronal activity rhythm in SCN slices from normal rats (Hamada et al., 1993), and with in vivo data showing that injection of VP does not phase shift the locomotor rhythm (Albers et al., 1984).

Finally, it appears that, despite being a highly circadian species, the SCN of the mink lacks any VP-immunoreactive neurones, although it does have the peptide in the supraoptic and paraventricular (PV) nuclei (Larsen and Mikklesen, 1993). This could be an interesting model with which to study VP function in the SCN.

Effects of reduced expression of VP in the SCN

In addition to the models of complete absence of VP described above, there are several examples of

reduced expression of VP which indicate its involvement in SCN neurobiology.

Ageing is associated with a decrease in the amplitude of circadian rhythms (van Gool and Mirmiran, 1986), and this may relate to a gradual 'winding down' of the biological clock. Indications of the changes in the organisation of the SCN which occur during ageing include, an increased number of degenerating neurones in the DM division of the rat SCN (Woods et al., 1993), and a simultaneous decrease in the number of VP-immunoreactive neurones in this region (Roozendaal et al., 1987). Although a similar decline in numbers appears not to occur in humans, comparison of the number of VP-immunoreactive neurones in the human SCN with time of death shows a clear circadian fluctuation in young subjects (<50 years) with a peak during the day, while older subjects (>50 years) show no such variation (Hofman and Swaab, 1994). Interestingly, in rats, both this age-related decline of the number of VP-immunoreactive neurones (Lucassen et al., 1995) and the amplitude of sleep-wake rhythms (Witting et al., 1993) can be prevented by increasing the intensity of the light stimulus.

We have recently been examining whether there are changes in the electrical activity rhythm expressed by aged animals. Our data have shown that, when maintained in constant light (LL), aged male Wistar rats (15–18 months) are capable of displaying a free-running rhythm of electrical activity, and that this can be re-entrained to 12 h light/12 h dark (LD) after a period of about 3 weeks. Furthermore, consistent with previous reports that in both aged hamsters (24 months: Watanabe et al., 1995) and rats (23–28 months: Satinoff et al., 1993) there is a decrease in the circadian rhythm of spontaneous activity of SCN neurones, we have observed a reduction in the light phase peak of activity compared to young (3–4 month) animals. More importantly however, when we divided the population of SCN neurones on the basis of their ability to respond to VP, this attenuation of activity was found to be selective for the neurones responsive to VP. This suggests that the age-related decline is either an inherent property of this subpopulation of neurones or that the afferent drive to these neurones was declining. In this respect, we found no significant difference in the proportion of VP-sensitive SCN neurones or in the magnitude of the VP-induced excitation, suggesting that by mid-age there has been no change in the expression of V_1 receptors. Therefore, we conclude that the declining expression of VP in the SCN underlies an age-related decrease in the power of the activity rhythm. Although the animals we have used are younger (15–18 months) than those shown to have effects on neurone number (>30 months: Roozendaal et al., 1987; Lucassen et al., 1995), it is quite possible that changes in the levels of expression of VP precede the changes in number of VP-immunoreactive neurones. However, it will be necessary to quantify mRNA levels in order to substantiate this conclusion.

A second model showing the importance of differential expression of VP in the SCN, is that of the inter-individual variation in circadian rhythmicity shown by male voles, a measure which is correlated with VP expression in the SCN (Gerkema et al., 1994). Under constant light conditions VP immunoreactivity is suppressed in the SCN of all animals, and this was associated with a complete loss of a circadian pattern of locomotor activity in some animals. Although this rhythm was regained when these animals were placed in LD this group of animals had a significantly higher number of VP neurones in the SCN than those which maintained rhythmicity throughout. A similar correlation has been observed in selected mouse lines in which a high amplitude rhythm of wheel running activity was associated with increased density of VP-immunoreactive neurones (Bult et al., 1993). However, in both these latter cases it remains to be determined whether these effects are mediated through changes in electrical activity rhythms or the output system of the SCN.

Transmitter interactions in the SCN

Although VP is the principal peptide found in the SCN, a large number of other transmitters are expressed in this nucleus (Inouye and Shibata, 1994) and can potentially participate in rhythm generation through interactions with VP-sensitive neurones. It has been demonstrated that a proportion of VP-sensitive neurones will also show exci-

tatory responses to NPY (Shibata and Moore, 1988) but, although neurotensin-binding sites have been detected in the VL division of the SCN (Francois-Bellan et al., 1992), we have not detected any effect of this peptide on VP-sensitive or -insensitive neurones (unpublished data). More recently we have been examining the main inhibitory transmitters in the SCN, somatostatin and GABA.

Double immunostaining has shown synaptic associations between VP axons and somatostatin perikarya and vice versa, suggesting a reciprocal relation between these two cell types (Daikoku et al., 1992). Our recordings show that application of somatostatin-14 causes dose-dependent and long lasting inhibition of a high proportion of SCN neurones (Fig.3; Ingram et al., 1995), and studies using the $SSTR_2$-selective agonist BIM 23027 has shown that this is the most predominant receptor sub-type in the SCN. Comparison of the effects in the different phases of the circadian cycle showed that a significantly greater proportion of neurones respond to somatostatin during the subjective dark phase compared to the light phase (48% vs. 79%). Furthermore, the pattern and magnitude of responses varied in the different phases of the cycle: inhibitions being significantly greater and of longer duration during the dark phase, suggesting either a rhythm of receptor expression or a variable interaction with endogenous peptide, similar to the situation for VP.

This circadian variation in sensitivity to somatostatin is important in view of the marked circadian changes in somatostatin synthesis and content in the SCN. The cycle of somatostatin content peaks at around CT 4 (Shinohara et al., 1991; Fukuhara et al., 1993) and somatostatin mRNA also fluctuates during the circadian cycle with a peak at CT 0 (Takeuchi et al., 1992). Neurones of the SCN express an identical 24 h rhythm of somatostatin-like immunoreactivity in both sighted and blind rats (Shinohara et al., 1991) and animals maintained under constant darkness (DD) (Fukuhara et al., 1993). In all cases the peak content at CT 4 which declines prior to lights off reaching a nadir at CT 16 during the subjective night. Unfortunately, it is not known whether this increased content during the light phase is accompanied by a reduction or increased in peptide release and, until this can be resolved, it will not be possible to determine the effect on the activity of SCN neurones.

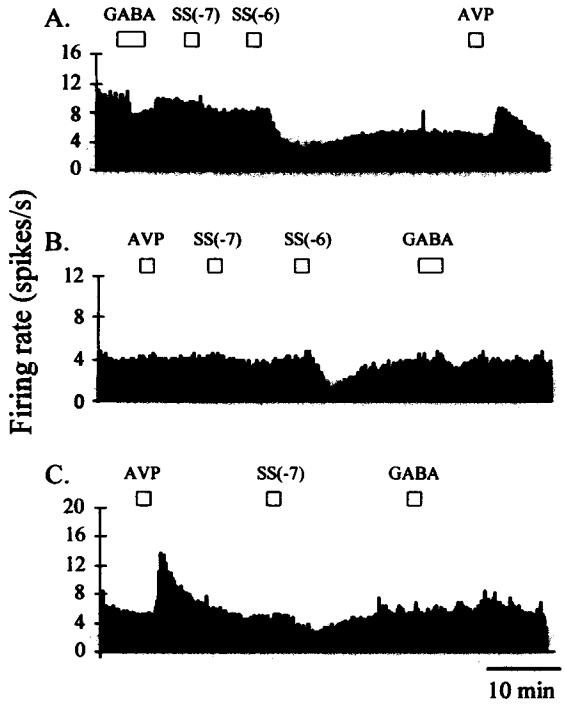

Fig. 3. Interaction between VP and inhibitory transmitters in the SCN. Somatostatin (SS) causes long duration and dose-dependent inhibition of SCN neurones particularly at 10^{-6} M (SS(−6)). This inhibition is both on neurones excited by 10^{-6} M VP (A,C) and neurones insensitive to VP (B). The effect of somatostatin is greater than that of GABA (10^{-4} M).

The inhibitory effects of somatostatin are likely to contribute to the phase regulating actions of the peptide. Previous studies have shown that somatostatin can cause either phase delays or phase advances to the rhythm of electrical activity depending on the time of administration (Hamada et al., 1993), while depletion of somatostatin with cysteamine caused only phase advances (Fukuhara et al., 1994). Although these authors showed that the phase shifting effect of somatostatin could be blocked by the purported antagonist, cyclo-(Pro-Phe-D-Trp-Lys-Thr-Phe), we have been unable to block the inhibitory effects of somatostatin with this compound (unpublished data).

In view of the observation that VP sensitivity

appears to characterise highly circadian neurones, we have looked for interactions between these peptides. While some somatostatin-sensitive neurones were excited by 10^{-6} M VP (Fig. 3A,C) others were without effect (Fig. 3B), suggesting at least two target populations. Furthermore, inhibition by somatostatin did not block the excitatory response to VP, indicating that they act through separate post-receptor mechanisms. Although the convergent effects indicates that somatostatin is likely to modulate the activity of those VP-sensitive neurones displaying the greatest circadian variation in firing rate, the ability of both peptides to affect firing rate but only somatostatin to effect phase changes (Hamada et al., 1993) strongly suggests that phase shifting is not mediated through simple changes in neuronal activity but may reside in the ability of second messengers to interact with a molecular oscillator.

The other main inhibitory transmitter in the SCN is GABA, and a large number of synapses and perikarya within the SCN are immunoreactive for the GABA synthesis enzyme glutamic acid decarboxylase (van den Pol and Gorcs, 1986). This is consistent with the mainly inhibitory synaptic potentials recorded in cultured SCN neurones (Welsh et al., 1995), and the ability of application of GABA to inhibit a large number of SCN neurones both in slices (Liou et al., 1990; Strecker et al., 1995) and organotypic culture (Bos and Mirmiran, 1993). We have examined the possible interaction between GABA and the neurones responsive to VP and/or somatostatin (Fig. 3). In agreement with previous studies (Liou et al., 1990), our data show that application of 10^{-4} M GABA has very rapid inhibitory effects although these rarely cause total suppression of neuronal activity (Fig. 3A,B) and not all neurones were affected (Fig. 3C). Comparison of the effects of somatostatin and GABA showed that the peptide was far more potent and long lasting than GABA. There was no direct correlation between responsiveness to either VP or somatostatin and responses to GABA, further suggesting that VP acts on a number of pharmacologically distinct populations of SCN neurones. Future studies will require that these populations become more accurately defined in order to elucidate their role in rhythm-generating circuitry.

Role of VP in the efferent pathways of the SCN

In addition to synapsing and releasing VP within the nucleus, the SCN is one of the major sources of central VP which can signal circadian rhythms to the rest of the CNS. This signal can take the form of a humoral signal in the cerebrospinal fluid (CSF), and measurement of VP levels in the CSF (Reppert et al., 1981; Mens et al., 1982; Schwartz and Reppert, 1985) and the SCN (Kalsbeek et al., 1995) show circadian patterns of release. However, although these are dependent on intact SCN there is no direct evidence to show that the peptide found in the CSF is of SCN origin. On the other hand, the output signal may be neuronal, and pathways have been described projecting principally to mid-line diencephalic areas (Watts and Swanson, 1987). This output function of VP may play an important role in determining circadian patterns of motor (Kruisbrink et al., 1987; Arnauld et al., 1989) and appetitive (Reghunandanan et al., 1987, 1992) behaviours. However, some of the best evidence for a functional role of extranuclear VP is in the transduction pathway controlling circadian activity of the hypothalamo–pituitary–adrenal (HPA) axis. Lesions of the SCN increase both basal and stress-induced HPA activity, abolishing the diurnal variation in plasma corticosterone concentration (Buijs et al., 1993). Infusion of VP into the dorsomedial hypothalamic/paraventricular region of SCN-lesioned animals will decrease the levels of corticosterone, while infusion of an antagonist into intact animals causes a marked increase (Kalsbeek and Buijs, 1992; Kalsbeek et al., 1992). Furthermore, analogous to our studies on the time-dependent effect of a V_1 antagonist on electrical activity (Mihai et al., 1994b), the magnitude of the effect of antagonist infusion varied with the circadian cycle, being greatest during the latter half of the light phase when endogenous VP secretion will have been maximal (Kalsbeek et al., 1996b). Finally, if the levels of endogenous VP were prevented from falling during the late light phase by exogenous infusion, then the normal rise in corticosterone seen at the light–dark transition was blocked (Kalsbeek et al., 1996a).

Thus, VP appears to fulfil the role of the circa-

dian inhibitor of HPA function, although it remains to be demonstrated whether this VP projection runs directly to the PVN and mediates some of the inhibitory and excitatory responses seen following SCN stimulation (Hermes and Renaud, 1993), or is mediated via the dorsomedial hypothalamic nucleus (Kalsbeek et al., 1996a). In either case it is unclear why microdialysis in the paraventricular/dorsomedial hypothalamic region fails to show a circadian rhythm of VP release (Kalsbeek et al., 1995).

Finally, it is interesting to note that, in respect of the transmission of circadian signals to the rest of the brain, it has recently been shown in hamsters that a diffusible factor produced by SCN grafts is able to impose strain-specific periodicity on the host (Silver et al., 1996). Whether this factor is (at least in part) VP remains to be determined.

Model for the role of VP in the SCN

The foregoing data suggest that it would be wrong to conclude, as others have (Inouye and Shibata, 1994), that VP is only an output mediator linking the circadian pacemaker to areas outside the SCN. Although this is one important function, VP also has a major function in the amplification of pre-existing circadian activity, and this function may have evolved as a mechanism to increase the biological significance of the circadian signal. This amplification function seems to involve a direct feedback excitation mediated through V_{1a} receptors and which is greatest during the subjective light phase when peptide release is maximal. The targets for this feedback include a number of sub-populations of SCN neurones, which appear to share a common characteristic in being highly circadian. Although the VP-producing cells of the DM division of the SCN receive recurrent synaptic inputs from other VP neurones, it is not clear to what extent these neurones are under tonic excitatory drive, especially since microdialysis data have shown that retrodialysis of a V_1/V_2 antagonist into the SCN does not modify VP release (Kubota et al., 1996). However, the electrical rhythmicity of these neurones may be secondary to the molecular rhythm of VP gene transcription.

Interestingly, VP-responsive SCN neurones include both a population excited by optic nerve stimulation and a population non-responsive to this stimulus (Shibata and Moore, 1988), supporting the hypothesis that VP will interact with both retinorecipient and non-retinorecipient neurones. In addition to these photic inputs which will play a role in entrainment to environmental light cues, VP-sensitive neurones also receive inhibitory inputs from somatostatinergic and GABAergic neurones (Fig. 4). These convergent inputs from inhibitory neurones may superimpose a further level of rhythmicity by the changing level of inhibition which is greatest during the subjective dark phase.

While dysfunction of the VP system of the SCN that occurs during ageing or in various genetic defects does not lead to an absence of circadian rhythmicity, there is a dampening of circadian activity due to the absence of the amplification mechanism, and a dysregulation of those systems (such as the HPA axis) which depend upon VP for the transduction of circadian signals. Future studies using better models of modified VP transmission

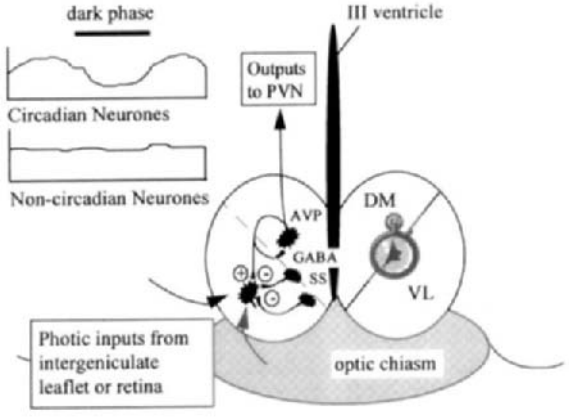

Fig. 4. VP neurones in the dorsomedial (DM) region of the SCN provide one of the main output systems from the SCN projecting to the paraventricular and dorsomedial hypothalamic regions. Recurrent connections both on the VP neurones themselves and on other retinorecipient neurones in the ventrolateral (VL) SCN provide a mechanism for amplification of circadian activity. This circadian activity is further modified by convergent inhibitory inputs from GABAergic and somatostatin (SS) neurones located in the intermediate zone of the nucleus.

will allow us to further define the role of VP in the SCN.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 4.3

The suprachiasmatic nucleus–paraventricular nucleus interactions: A bridge to the neuroendocrine and autonomic nervous system

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Vasopressin (VP) is one of the principal neurotransmitters of the suprachiasmatic nucleus (SCN). By means of anatomical, physiological and electrophysiological techniques we have demonstrated that VP containing pathways from the SCN serve to affect neuroendocrine and 'autonomic' neurons in the paraventricular nucleus. By direct and indirect connections VP serves to inhibit corticosterone secretion, not only by affecting ACTH secretion but also by controlling the adrenal cortex via a neuronal route. Apart from controlling the pineal and adrenal, we also observed that the SCN is able to influence the heart. Subjecting rats or humans to light affects heart rate in a dose-dependent manner. These results suggest an important role for the SCN and VP in the SCN in the regulation of neuroendocrine and autonomic functions.

Introduction

Life on our planet owes its existence to the presence of the sun. The rotation of the earth around its axis exposes all but permanently cave-dwelling organisms to the daily sunlight. These circumstances make it understandable that most organisms have somehow organized their life around this 24-h light/dark cycle. In order to facilitate this organization, most organisms have, in the course of evolution, developed structures that have a clock-like function.

Often these structures are characterized by their ability to maintain a circadian, i.e. nearly 24 h cycle of activity and inactivity. In mammals this has resulted in the development of the suprachiasmatic nucleus (SCN). This is a tiny structure of approximately 10 000 cells in rats, and approximately 70 000 cells in humans (Hofman et al., 1993; Swaab et al., 1996). It is quite conveniently located, on top of the optic chiasm, from which it receives direct information about the light/dark cycle from the retina. A series of experiments in the last quarter of a century proved that this SCN is the endogenous master oscillator in mammals (Moore and Eichler, 1972; Ralph et al., 1990).

In animals, mostly hamster and rat, lesioning of the SCN obliterated all capacity for synchronizing their activities with the daily light/dark cycle (Moore and Eichler, 1972). Transplantation of an SCN of a young animal to the third ventricle of such SCN lesioned animals restored their circadian rhythm in the sense that it resulted in the original activity pattern of the donor animal (Ralph et al., 1990).

SCN neurons were shown to have a higher level of electrical activity during the light period as compared to the dark period, also without an actual change in the lighting conditions. The endogenous oscillator properties of this structure were proved by the fact that neurons of the SCN maintained this capacity, even in vitro (Groos et al., 1983; Bos and Mirmiran, 1990; Welsh et al., 1995).

Also in humans there is compelling evidence that the SCN has a function as an endogenous oscillator, since lesions or tumors in this area disrupt the ability of these persons to synchronize their activity to the light/dark cycle (Cohen and Albers, 1991).

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SCN properties

Beside endogenous clock functions that have a period of nearly 24 h, it is essential that the clock can be synchronized to the changing light/dark cycle. The most important entrainment pathway for this synchronization is the input from the eye that reaches the SCN direct, by way of the retino-hypothalamic tract (RHT) (Morin, 1994). This RHT reaches a particular set of neurons in the SCN, mostly in its ventral part. This RHT is not only present in rodents, where most phase-shifting studies by light have been conducted, but also in human, where anatomical and functional evidence has been found for the existence of this RHT (Lewy et al., 1980; Dai et al., 1998).

In most animals, light affects the SCN in a complicated manner. However, experiments are mainly conducted in nocturnal animals. During constant darkness light does not influence the activity in the subjective light phase, but it does phase-delay the behavioral activity in the beginning of the subjective dark phase, while it phase-advances the activity at the end of the dark phase (for review see Morin, 1994). This coincides with a variable pattern in immediate early gene (c-fos) induction: no induction of fos in the inactivity (the subjective light) period and a localization of c-fos in a different set of neurons, depending on the time of exposure to light in the dark phase (Romijn et al., 1996). In addition, light results in an immediate inhibition of melatonin in the dark phase, irrespective of when the light is given: in the beginning, middle, or end-phase of the dark period (Nelson and Takahashi, 1991).

One of the first demonstrations of the endogenous activity changes in the SCN was by means of the uptake of deoxyglucose, which was clearly associated with the subjective light phase. Much more deoxyglucose is taken up by the SCN in the (subjective) light period which coincides with the period that the SCN shows its highest electrical activity (Schwartz and Gainer, 1977; Gillette and Reppert, 1987). These experiments also showed this pattern of deoxyglucose uptake, irrespective of whether nocturnal or diurnal animals were examined.

This indicates that activity of the SCN may have a completely different function in day active animals as compared to night active ones. In day active animals the activity of the SCN transmits the signal to activate behavior, while the opposite is true for night active animals.

Recently, we observed that this is also true for light; light exposure results in a decrease of activity and heart rate in nocturnal animals, while in humans the reverse is observed (Buijs et al., unpublished observations).

Neurotransmitters of the SCN

Not long after the discovery that the SCN was responsible for the capacity of the animal to synchronize its activities to the daily light/dark cycle, the first peptide was demonstrated in the neurons of the SCN. In their anatomical analysis of the classical neuroendocrine neurons of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, first neurophysin and later vasopressin were demonstrated to be present in the neurons of the SCN by Swaab et al. (1975) and Vandesande et al. (1975).

Soon after, a host of peptides was demonstrated inside the neurons of the SCN (Card et al., 1981; Vandenpol and Gorcs, 1986), to which only much later GABA and glutamate were added as potential transmitters of the biological clock (Buijs et al., 1994; Hermes et al., 1996). Interestingly, the first transmitter molecule to be demonstrated in the SCN also appeared to be the first peptide to be demonstrated to have a clearly fluctuating level with a circadian rhythm, i.e. high in the light phase and low in the dark phase (Gillette and Reppert, 1987). Lesioning studies demonstrated that only removal of the SCN resulted in the loss of this circadian pattern in vasopressin levels in the CSF, while in addition it resulted in a drop in vasopressin CSF level to an almost undetectable level (Schwartz and Reppert, 1985; Jolkonen et al., 1988). The latter observation indicates that the SCN not only drives the circadian rhythm in vasopressin secretion, but is also the main source of CSF vasopressin. Other studies have demonstrated, in addition, that the concentration of vasopressin mRNA also showed a daily fluctuation, together with a daily change in the length of the poly-A tail of the vasopressin

mRNA (Uhl and Reppert, 1986; Robinson et al., 1988). An interesting observation was made in the Brattleboro rat, which is unable to synthetize vasopressin due to a deletion in its vasopressin coding part of the DNA. Here, this mRNA, transcribed on the mutated DNA, still shows a daily variation in its synthesis (Uhl and Reppert, 1986).

Several studies have demonstrated that SCN vasopressin levels are affected by the concentration of adrenal steroid hormones. Adrenalectomy as well as treatment of the rats with dexamethasone affected the levels of vasopressin or vasopressin mRNA in the SCN (Larsen et al., 1994).

These data indicate an important role of glucocorticoids on the SCN. Interestingly, glucocorticoid receptors are only demonstrated in the SCN early in development and disappear after 5 days postnatally (Rosenfeld et al., 1993). For melatonin no such effects have been described, while it is certain – by the presence of melatonin receptors in the SCN and their effect on the electrical activity on SCN neurons (Reppert et al., 1994; Liu et al., 1997) that melatonin will affect the SCN noticeably.

SCN projections

After the discovery of the presence of vasopressin within SCN neurons, the description of the vasopressin pathways emanating from the SCN followed (Buijs, 1978; Sofroniew and Weindl, 1978). Initially this was purely done on the basis of following stained fibers, or even on the basis of the size of VP fibers in different brain regions. Lesioning the SCN resolved this issue and clarified by means of disappearance of vasopressin fibers in several target areas (Hoorneman and Buijs, 1982), in the vasopressin-containing SCN projections. These vasopressin-containing SCN projections were demonstrated to run to the area of the PVN, to the DMH, to the paraventricular nucleus of the thalamus (PVT) and to the organum vasculosum of the lamina terminalis. In the mean time anterograde tracing studies by means of HRP were carried out as well, but these did not allow determination of the SCN projections in more detail (Berk and Finkelstein, 1981; Stephan et al., 1981).

Finally, by means of precise, localized injections of the anterograde tracer Phaseolus vulgaris leucoagglutinin (Pha-L), it proved possible to determine the general projections of the SCN (Watts et al., 1987; Buijs et al., 1994; Vrang et al., 1995). With respect to the different peptides that are located in different regions of the SCN, some small differences in projection patterns and densities were described for VIP and vasopressin (Buijs et al., 1993). Especially the Pha-L tracing studies, but also the VIP staining, revealed a more detailed picture of the innervation of the PVN. It became clear that SCN projections in general avoid the body of the PVN, except for its ventral periventricular and dorsal borders. In the dorsal part of the PVN, where mostly neurons are situated that project to the spinal cord, many contacts between SCN projections and these so-called autonomic PVN neurons have been demonstrated (Teclemariam-Mesbah et al., 1997a,b).

The exact vasopressin projections to the PVN, however, have remained obscure, mainly because of the interference with vasopressin fibers of PVN origin.

SCN–hypothalamus interaction

Taken together all animal tracing studies indicate that the observed SCN projections are mainly restricted to hypothalamic target areas with the exception of its projections to the paraventricular thalamus (PVT), and the lateral geniculate nucleus (Fig. 1). On the basis of these anatomical data alone, it is clear that these hypothalamic projections of the SCN will serve to synchronize homeostatic functions with the daily light/dark cycle. On the basis of what is already known of these hypothalamic targets with respect to projections, transmitter content and physiological experiments a further subdivision can be made with respect to specific SCN functions. We therefore suggested in Fig. 1 which functions might be influenced by the SCN in these different areas. It seems evident that the SCN projections to the various sites in the medial hypothalamus will serve to modulate the release of hormones into a pattern which expresses a circadian rhythm. Most pituitary-controlling neuroendocrine neurons are situated in the medial hypothalamus. However, anatomical evidence that the SCN has a direct interaction with all neuroendocrine neurons

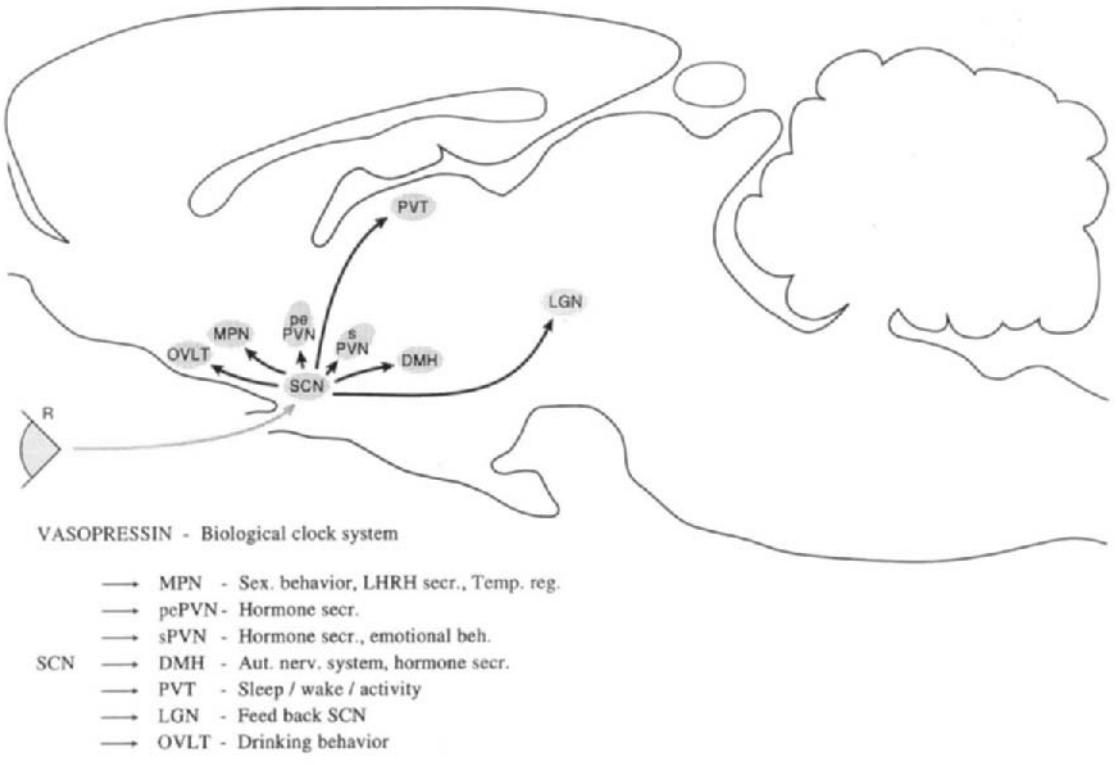

Fig. 1. Illustration of a sagittal scheme of the rat brain with the main projections of the suprachiasmatic nucleus (SCN). Under the scheme is indicated which functions might be influenced by the SCN in that particular target area. MPN, medial preoptic nucleus; pePVN, periventricular part of the paraventricular nucleus of the hypothalamus; sPVN, subPVN area; DMH, dorsomedial hypothalamus; PVT, paraventricular nucleus of the thalamus; LGN, lateral geniculare nucleus; R, retina.

in the hypothalamus is not available yet. Consequently, closer examination of the precise SCN projections in combination with target identification will have to provide the picture that reveals where exactly the SCN may influence these releasing factor containing neurons. In relation to the control of general (motor) activity it is not apparent yet what target area is involved. It is possible that this message is transmitted to all SCN target areas, and that all these target areas together control to some extent motor activity for a particular kind of behavior. On the other hand, it is possible that one or more target areas, such as the PVT or LGN, serve to modulate motor activity more precisely (Berendse et al., 1988; Johnson et al., 1988). Consequently, it is of importance to also collect knowledge on the projections and functions of the target sites of the SCN, thus enabling conclusions on the processes that may be influenced by these target areas.

In order to acquire more insight into the manner in which the SCN influences corticosterone secretion, considerable attention was paid in our anatomical and physiological studies to elucidate sites and action of SCN transmitters. Initially a great deal of effort was directed to the question whether the observed anatomical network of SCN terminals in the dorso-medial hypothalamus can indeed provide an explanation for the diurnal peak in corticosterone.

Vasopressin co-localization with GABA

One of the intriguing questions regarding the co-localization of the various peptidergic transmitters of the SCN with GABA (Buijs et al., 1994, 1995) is

whether the neuron is able to control which transmitter will be released from its terminal. Numerous studies have appeared indicating that a peptide is more efficiently released with bursts of activity and at a higher frequency than the classical transmitters which are already liberated with single neuronal discharges (Lundberg et al., 1981, 1994; Whim and Lloyd, 1989; Cropper et al., 1990). This suggests that during the light period, when the SCN neurons are most active (Inouye and Kawamura, 1979; Gillette and Reppert, 1987), a larger portion of peptides is secreted by these active neurons, whereas during the dark period the secretion of GABA prevails. The high level of VP released by the SCN during the (subjective) light period (Earnest and Sladek, 1986) seems to support this idea. This suggests that the function of a single SCN neuron containing VP and GABA in its terminal may change depending on its firing frequency: excitatory during the (subjective) light period, when VP is released (VP is usually found to be excitatory; Joëls and Urban, 1982; Raggenbass et al., 1989; Van den Hooff and Urban, 1990) switching to inhibitory during the dark period, when GABA is released. Here, however, we are faced with another problem: if GABA is also released during the light period, its inhibitory action would be in contrast with the presumed activation induced by VP. It is clear that electrophysiological studies examining post- and presynaptic actions of GABA and VP are necessary for understanding the functional significance of this co-localization. Electrophysiological studies by Hermes and Renaud (1993) have indeed indicated that stimulation of the SCN may result in different responses in target structures of the SCN.

In conclusion, the wealth of intrinsic connections containing GABA suggests that within the SCN powerful mechanisms are available to shut down the activity of this nucleus. In addition, as was noted by Vandenpol and Dudek (1993), a system of self-inhibiting oscillators may provide for the necessary stability, but it may also explain phenomena such as synchronous firing. This may then be another mechanism for synchrony since Bouskila and Dudek (1993) showed that even without calcium dependent synaptic transmission SCN neurons display synchronous multi-unit activity. The function of GABA-GABA axo-axonal contacts observed by Buijs et al. (1995) indicates a presynaptic inhibitory regulation which may serve to lessen this excessive inhibitory drive within the SCN. Also the fact that not every neuron expresses GABA in its terminals (or in all of its terminals) provides room for activational mechanisms within the SCN. In order to solve the questions related to the functional significance of the intrinsic connections in the SCN and the properties of the network of peptidergic and peptide/amino acid connections it will be necessary to clarify the anatomical circuits within the SCN and to investigate the molecular and cellular mechanisms of activation and inhibition of these circuits. Finally, theoretical studies that will make use of these accumulated anatomical and physiological data are required to provide new and testable hypotheses regarding the manner in which the SCN may express its different circadian properties.

Anatomical and physiological basis for diurnal corticosterone rhythm

Lesions studies indicated that the SCN has a profound inhibitory influence on corticosterone secretion, i.e. an SCN lesion results in highly elevated plasma corticosterone levels after a novel environment stimulus as compared with intact animals which only show a moderate increase in corticosterone levels irrespective of the circadian time at which they are subjected to a novel environment (Buijs et al., 1993b). Since the corticotrophin release hormone (CRH) producing neurons in the parvocellular part of the PVN (PVNp) control for a large part the adrenocorticotropin releasing hormone (ACTH) release from the adenohypophysis, which in turn stimulates the adrenal cortex to secrete corticosterone (Swanson and Simmons, 1989; Dallman et al., 1992), much attention has been directed to the question whether the SCN is able to influence these CRH neurons directly. Thus far, in spite of the observed circadian rise in CRH mRNA immediately preceding the corticosterone peak (Kwak et al., 1993), all anatomical studies have not indicated any substantial direct projection to these neurons (Buijs et al., 1993; Vrang et al., 1995). Instead, Watts et al. (1987) demonstrated a

massive projection of the SCN to an area just ventral of the PVN, which they called the sub-PVN zone. In their paper they suggested that this region may project into the PVN. Furthermore, using Pha-L tracing, Buijs et al. (1993) showed that not only the subPVN but also the DMH receives a substantial SCN input. Subsequently Roland and Sawchenko (1993) provided evidence that these peri-PVN areas contain GABAergic neurons projecting to parvocellular PVN neurons. This also holds for the DMH, which has an established direct connection with the PVNp; one of the peptide transmitters of this projection is galanin (Ter Horst and Luiten, 1986; Levin et al., 1987). Moreover, Kalsbeek et al. (1992) provided substantial evidence that the DMH is the site, or one of the sites, where VP of SCN origin serves to inhibit corticosterone and ACTH secretion. This corroborates with the observation that the SCN projects extensively to the DMH with VP fibers (Hoorneman and Buijs, 1982).

Because of the lack of direct contacts between SCN efferent and PVN-CRH neurons and in order to investigate which (other) putative sites in the hypothalamus might be influenced by the SCN and may change the HPA axis, we decided to label SCN efferents in combination with identification of neurons implicated in the stress response by fos immunocytochemistry (Buijs et al., 1993). A 15-min restraint stress resulted in the presence of numerous fos-positive neurons in the medial hypothalamus. Apart from the CRH neurons in the PVNp, fos-positive neurons were also present in the DMH, periventricular PVN (PVNpe), dorsal cap of the PVN (PVNdc) and rostral PVN (PVNr). All these areas with fos-positive neurons receive a dense input from the SCN except for the PVNp, where only a few Pha-L-labeled SCN efferents could be detected suggesting no or a very limited output to CRH neurons. Similarly, when we employed a CRH-immunocytochemical staining to identify the PVN neurons involved in the control of the ACTH cells of the hypophysis, we were also unable to demonstrate extensive interaction between SCN efferents and CRH neurons. This observation was corroborated by Vrang et al. (1995), who used the same procedure. In view of (1) the elaborate SCN terminals in the DMH region on 'after stress fos-positive' neurons and the extensive projections of the DMH to the parvocellular PVN (Ter Horst and Luiten, 1986), (2) the physiological studies pointing to the DMH as an important target for the SCN to control corticosterone secretion (see Kalsbeek et al., 1996a,b), (3) the 'after stress fos-positive' neurons that receive SCN input are located around the PVN in areas that project into the PVN; all anatomical and physiological evidence so far points to an important indirect action of the SCN onto CRH neurons. The disadvantage of the anatomical approach thus far is that it does not allow the determination of contacts between SCN fibers and dendrites of PVN neurons. Therefore an in vitro slice preparation and electrophysiological analysis were used to investigate the functional connectivity between SCN and PVN.

Functional connectivity between rat SCN and PVN

This matter was addressed by evaluating the effects of electrical microstimulation in SCN on the excitability of PVN cells, as measured by extracellular recording techniques. Investigations were performed in an anesthetized (using pentobarbital) whole animal preparation that enables the identification, by means of antidromic activation, of known categories of PVN cells, according to their projections to the posterior pituitary (PP), median eminence (ME), or dorsomedial medulla (Swanson et al., 1980). Briefly, PVN neurons that project to the PP are magnocellular neurosecretory neurons that synthetize either vasopressin or oxytocin for release into the general circulation and subsequent action on kidney and uterus, respectively. ME-projecting PVN cells comprise parvocellular neurosecretory neurons that differentially express immunoreactivity for various peptidergic releasing factors that, following release from ME, act on the anterior pituitary gland to regulate release of hormones acting on endocrine glands. A subpopulation of these cells produces corticotropin-releasing factor (CRF) and controls, through anterior pituitary release of adrenocorticotrophic hormone (ACTH), the secretory activity of the adrenal gland (i.e. corticosterone secretion). Lastly, PVN

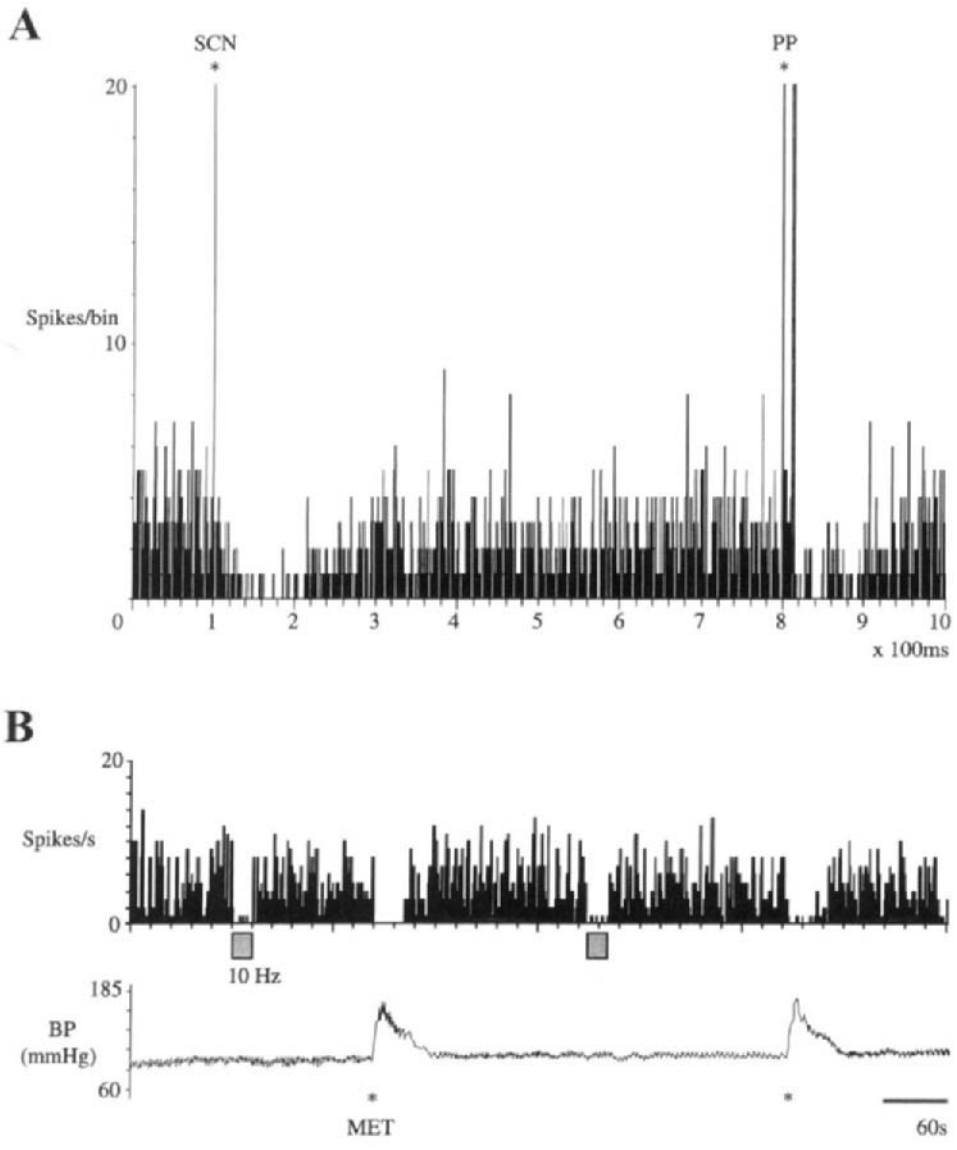

Fig. 2. (A) Example of a post stimulus histogram (PSH) of a paraventricular neuron projecting to the PP showing the typical reduction in excitability of such neurons following 1 Hz stimulation of the SCN. (B) Rate meter record of a putative magnocellular vasopressin neuron (upper trace) demonstrating a cessation of activity following a metaraminol-induced (MET; asterisk) rise in arterial blood pressure (BP; lower trace). Stimulation of the suprachiasmatic nucleus at higher frequencies (10 Hz) induced an arrest of spontaneous firing of the neuron (upper trace; antidromic spikes visible).

neurons innervating brainstem and spinal cord are in a position to influence parameters regulated by the autonomic nervous system, such a blood pressure and heart rate, and pineal melatonin secretion.

Our observations indicate that electrical stimulation in SCN influences, in a differential manner, the excitability of all cell categories in PVN (Hermes and Renaud, 1993). The majority of neurons, including magnocellular neurosecretory cells (Fig. 2), showed a decrease in firing following SCN

stimulation. The influence of SCN on these latter neurons is remarkable in view of the very sparse innervation by SCN efferents of the magnocellular subdivision of the PVN (Watts et al., 1987), although may be expected in view of evidence for circadian rhythmicity in the activity of the magnocellular neurosecretory system (Windle et al., 1992).

Resolving issues of functional connectivity between SCN and PVN, this study has raised new questions with respect to the neuropharmacology of SCN-PVN connections. First, short latency inhibition following SCN stimulation suggests that the responses are mediated, directly or indirectly, by GABA, a dominant fast inhibitory transmitter in the hypothalamus (Decavel and Van den Pol, 1990). In contrast, late and prolonged excitation following SCN stimulation indicates a sustained membrane depolarization, which can have various underlying mechanisms.

Synaptic mechanisms underlying SCN influence on PVN

To investigate underlying synaptic mechanisms of PVN responses to SCN stimulation by intracellular recording techniques, an in vitro rat hypothalamic slice preparation was developed that preserves projections from the anteroventrally situated SCN to the dorsocaudally located PVN. Instead of using antidromic activation as distinguishing criterium for different cell categories in PVN, cell types could be differentiated and partially identified on the basis of their intrinsic electrical membrane properties, recognized from spontaneous action potentials and by application of protocols injecting positive or negative current pulses through the recording electrode into the cell, to generate current–voltage (*I–V*) relationships (Fig. 3). In addition, intracellular labeling with biocytin provided morphological details of the different PVN cell types (Fig. 4). In agreement with results by Tasker and Dudek (1991) and Hoffman et al. (1991), type I neurons were identified as magnocellular neurosecretory neurons (see also Renaud and Bourque, 1991), and type II and III neurons as parvocellular cells.

The results on SCN-evoked responses in PVN

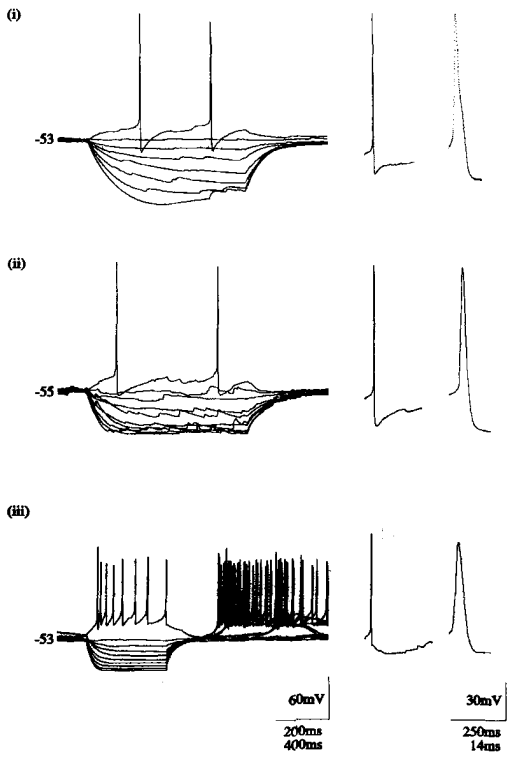

Fig. 3. PVN neurons display distinct electrical properties. The left column shows voltage responses to depolarizing and hyperpolarizing current steps. Current pulse protocol was −30 to 0 pA, 5 pA increments and +10 pA for type; −40 to 0 pA, 5 pA increments and +10 pA for type II and III. Duration of current pulse was 600 ms. Right column shows spontaneous action potentials at a slow and a fast time base. Both intracellular sharp electrode and whole-cell patch-clamp recordings (as shown here) revealed similar qualitative differences between PVN neurons. (A) Type I magnocellular neurons display a depolarizing sag (i.e. time-dependent inward rectification) and a delayed return to baseline membrane potential (caused by a transient outward conductance; Bourque, 1988) during and following membrane hyperpolarization, respectively. A shoulder is observed on the repolarizing phase of their action potentials. (B) Type II parvocellular neurons demonstrate pronounced instantaneous inward rectification and small low-threshold potentials (not shown). (C) Type III parvocellular neurons show large low-threshold potentials, observed here as depolarizing overshoots supporting bursts of action potentials following transient hyperpolarization. Their action potentials show a fast and a slow postspike hyperpolarization. Their action potentials show a fast and a slow postspike hyperpolarization.

neurons can be summarized as follows (Hermes and Renaud, 1993; Hermes et al., 1996).

1. In type I magnocellular neurosecretory neurons, electrical stimulation in SCN induced short latency inhibitory postsynaptic potentials (IPSPs) that reversed polarity at the chloride equilibrium potential and were mediated by $GABA_A$ receptors, since they could reversibly be abolished by the $GABA_A$ receptor antagonist bicuculline methiodide (BMI; Fig. 5A). The responses correspond to the short-latency, transient decrease in excitability observed in extracellular recordings in vivo from PVN neurons that could be unequivocally identified by antidromic activation of their PP axon terminals (Hermes and Renaud, 1993).
2. Detailed analysis of type I neurons revealed that responses were specific to stimulation within the SCN and probably evoked through monosynaptic pathways, since they neither changed their onset latency with increasing intensities of the SCN stimulus, nor showed failures following high frequency stimulation (Fig. 5A–D). Moreover, IPSPs were unaffected by the *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptor antagonists D-APV and CNQX. This latter result indicates also that evoked IPSPs were not generated disynaptically, i.e. through SCN glutamatergic input to local GABAergic neurons.

A potential site for a direct interaction between SCN axons and PVN neurons may be the periventricular subnucleus of PVN. As illustrated in Fig. 4,

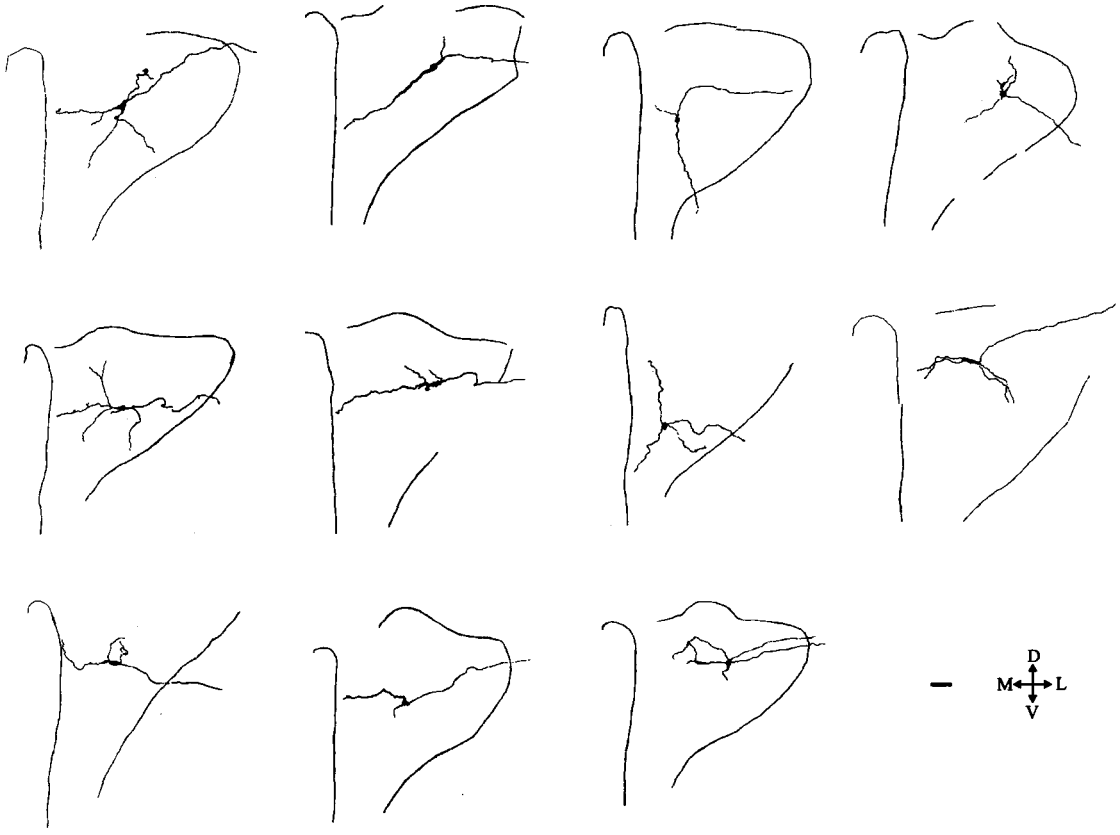

Fig. 4. Most type I PVN neurons have a dendrite that extends into the periventricular subnucleus. Camera Lucida drawings of the morphology of 11 type I neurons labeled by intracellular injection of biocytin. Axons (shown at a thickness similar to the dendrites) exit the PVN in a lateral direction. D, dorsal; V, ventral; M, medial; L, lateral.

a majority of type I neurons displays one or more medially projecting dendrites to this part of the PVN, thereby appearing the only site both covered by processes of these neurons and by SCN efferent fibers (Watts et al., 1987; Buijs et al., 1994).

Collectively, the data indicate the existence of rapid monosynaptic transmission from SCN to PVN carried by the amino acid transmitters GABA and glutamate. The results showing SCN-evoked PVN responses mediated by the amino acid GABA provide the first direct evidence for a role for this amino acid in synaptic transmission from the SCN, which was not unexpected in view of abundant immunoreactivity in the SCN and efferent fibers (Okamura et al., 1989; Francois-Bellan et al., 1990; Buijs et al., 1994). The indication for a possible role for glutamate in SCN efferent neurotransmission is more difficult to substantiate given the apparent difficulty to demonstrate anatomically its presence in SCN efferents. By excluding the possibility that stimulation of fibers of passage from optic chiasm (Johnson et al., 1988; Levine et al., 1991) or of surrounding regions underlies the excitation (through stimulation of other regions of the hypothalamus, including the optic chiasm) we suggest that activation of SCN neurons is responsible for the depolarizing responses induced by electrical stimulation (Fig. 5).

Although we have demonstrated here that the amino acids GABA and glutamate are implicated in SCN efferent neurotransmission, we have no evidence to support a neurotransmitter role of any of the peptides present in the SCN, despite strong indication from immunocytochemical and physiological studies (Vandesande et al., 1975; Card et al., 1981; Kalsbeek et al., 1992, 1996). Peptides, in contrast to amino acids, may be preferentially released from SCN axon terminals at times of increased neuronal activity (Earnest and Sladek, 1986; Cropper et al., 1990), and, therefore, single shock electrical stimulation may not be sufficient for their release. Moreover, a great deal of the SCN influence on PVN may flow through pathways using multiple synapses, i.e. through the subPVN, despite our demonstration of (additional) monosynaptic connections. Such a configuration may hinder the detection of peptide-mediated events. A major part of our future efforts will be aimed at

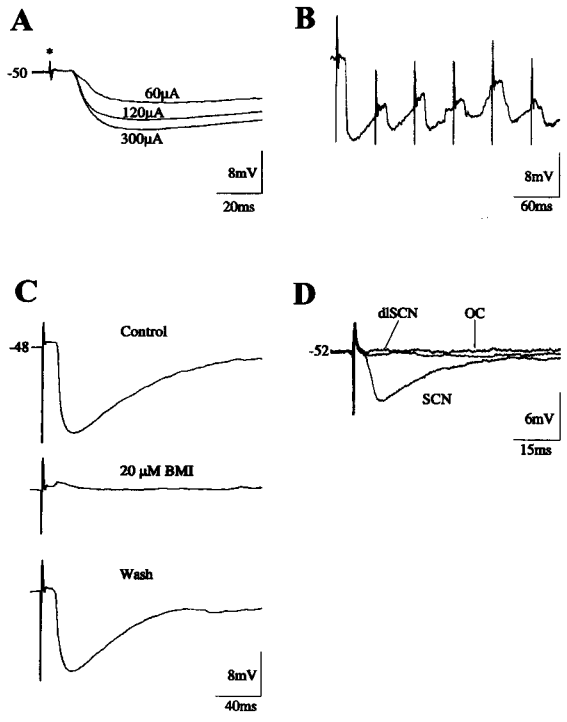

Fig. 5. SCN stimulation evokes monosynaptic IPSPs in type I PVN neurons. (A,B) SCN-evoked IPSPs in type I magnocellular neurons do not show shifts in latency with graded increases in stimulus intensities applied to SCN (*, stimulus; A, patch recording), or failures with high frequency stimulation of SCN (20 Hz; B, sharp electrode recording), suggesting mediation by monosynaptic pathways. Traces in (A) and following figures represent a mean of 10. (C) Evoked IPSPs in control media are reversibly abolished by the application of the $GABA_A$ receptor antagonist BMI (20 μm), leaving no residual effect (patch recording). (D) Superimposed records illustrate an IPSP evoked from stimulation in the ipsilateral SCN, whereas no response followed stimulation dorsolateral (dlSCN) or ventral from SCN in the optic chiasm (OC; sharp electrode recording).

pursuing the role these transmitters may play in regulating PVN excitability. Another approach we used to examine the role of peptides such as vasopressin in the transfer of circadian information to the PVN was by means of in vivo microdialysis experiments. Vasopressin was selected because it was shown to be released with a clear circadian pattern from SCN terminals, suggesting that such

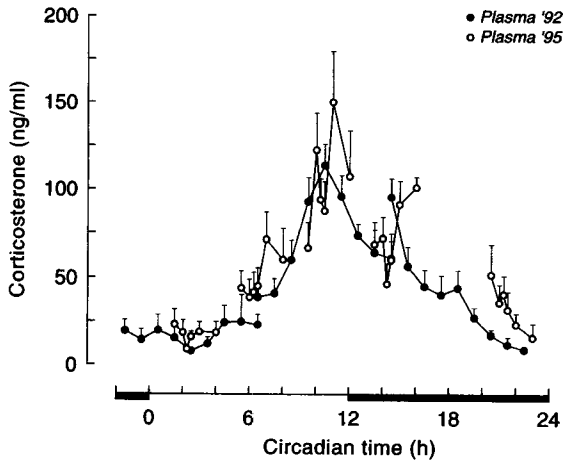

Fig. 6. Plasma corticosterone values (mean ± SEM) during Ringer's dialysis at different CT times (i.e., Plasma '95) compared with a previously established plasma corticosterone curve (i.e. Plasma '92) in control animals without an intracerebral probe. With permission from Kalsbeek et al. (1996).

a pattern in peptide secretion might be responsible for diurnal rhythms in corticosterone secretion (Fig. 6). Logically the PVN/DMH area was chosen as the brain site where this transfer of information from the SCN to the hypothalamo-pituitary axis (HPA) may take place.

The physiological basis of the circadian rhythm in plasma corticosterone levels

Inhibitory effect of vasopressin

Circadian fluctuations in circulating glucocorticoid levels have been reported for many species. In nocturnal animals such as the laboratory rat, plasma glucocorticoid levels are high at the onset of darkness and then decline, reaching a nadir in the morning. Our initial experiments using microinfusions in the PVN/DMH area showed a strong inhibitory effect of exogenous VP, but not VIP, on the release of corticosterone (Kalsbeek et al., 1992). The inhibitory effect of VP was only evident in SCN-lesioned animals, but not as clear as in intact animals. These results suggested an inhibitory effect of VP released by SCN terminals in the PVN/DMH area on the activity of the HPA-axis. The inhibitory effect of endogenously released VP on HPA-axis activity was confirmed by infusion of the VP V_1-antagonist d(CH_2)Tyr(Me)-AVP (i.e. Manning compound; Manning and Sawyer, 1984) in the PVN/DMH area of intact animals. Infusions were performed during the middle of the light period, i.e. when the secretory activity of VP-containing SCN neurons is maximal and corticosterone levels are low. Infusion of the VP-antagonist, but not the Ringer vehicle, caused an immediate and pronounced increase of circulating corticosterone levels (Kalsbeek et al., 1992). In SCN-lesioned animals the infusion induced a small increase of corticosterone secretion, but here Ringer and the V_1-antagonist were equally effective.

The above results indicated an inhibitory effect on the HPA-axis of endogenous VP, released from SCN terminals at the level of the PVN/DMH-area, during the middle of the light period. These initial data were further substantiated by microdialysis mediated administration of VP and its V_1-antagonist using different dosages. Stress-free infusion of the VP V_1-antagonist in the dorsomedial hypothalamus of freely moving, undisturbed animals during the middle of the light period (i.e. the trough of the corticosterone rhythm), caused an immediate dose-dependent increase of circulating plasma corticosterone. On the other hand, similar infusions of VP at the end of the light period completely prevented the diurnal rise in plasma corticosterone (Kalsbeek et al., 1996b). However, in all of the above studies only single time points were investigated. Therefore, to further specify the nature of the endogenous VP signal, intracerebral infusions of VP-antagonist were performed at different times of the day/night cycle. One hour infusions of the VP-antagonist were performed using in vivo microdialysis at CT2, 6, 10, 14 and 21. Corticosterone levels collected during Ringer infusions revealed a basal pattern of corticosterone release that was very similar to the one previously reported (Fig. 6). On the other hand, infusion of V, VP-antagonist caused pronounced increases of circulating corticosterone levels at CT6 and 10, whereas much smaller

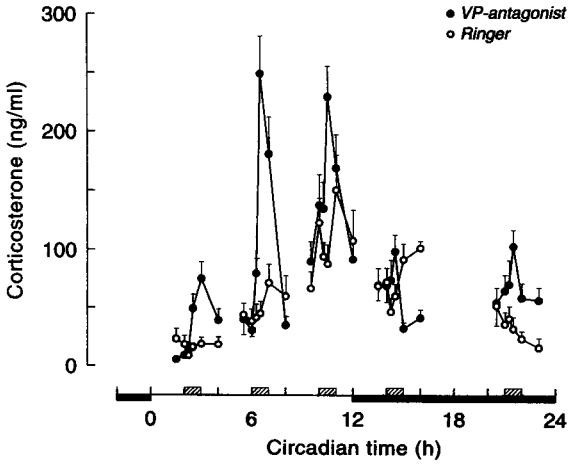

Fig. 7. Plasma corticosterone values (mean ± SEM) during Ringer's or VP-antagonist administration in the PVN/DMH area. Hatched boxes indicate the timing of the 1 h period of VP-antagonist administration. With permission from Kalsbeek et al. (1996).

increases were seen at CT2 and 21 (Fig. 7), evidencing the inhibiting nature of the VP projection.

The inhibitory control of the SCN-derived VPergic projection during the diurnal trough of adrenal activity is in agreement with the main inhibitory effect of the SCN on HPA activity as revealed by SCN lesions (Abe et al., 1979; Szafarczyk et al., 1979; Watanabe and Hiroshige, 1981; Buijs et al., 1993). In this respect, there is a remarkable correlation between the age-related elevation of basal HPA secretory activity during the diurnal trough (Sapolsky, 1992) and the pronounced decline of VP activity in the SCN during aging (Roozendaal et al., 1987). A similar phenomenon is observed in aging humans, i.e. higher basal levels of cortisol (Dodt et al., 1994; Seeman and Robbins, 1994; Ferrari et al., 1995) together with a decreased presence of VP in the SCN (Swaab et al., 1985; Hofman and Swaab, 1994). In addition, daily and seasonal rhythms of VP activity in SCN neurons may disappear with increasing age (Hofman et al., 1993), enhancing the deterioration of hormonal rhythms. Though correlative, these data strongly suggest that degeneration of the SCN (and more specifically its VP-containing population of neurons) with aging, is an important causal factor for elevated cortisol levels in elderly people.

Stimulatory SCN input to the HPA-axis

The differential effects of infusions of V_1-antagonist on plasma corticosterone levels depending on the time of the day (Fig. 7) provide further evidence for the rhythmic nature of the endogenous VP signal, with the peak release occurring during the light period. The variation in corticosterone responses upon application of the VP-antagonist, however, also illustrates that the VP-rhythm alone cannot explain the complete pattern of corticosterone responses. Therefore, in addition to the inhibitory control by VP, there is also a stimulatory SCN input to the HPA-axis, as discussed previously by us (Kalsbeek et al., 1992) and others (Cascio et al., 1987), which is active during the second half of the light period. Thus, the lack of a pronounced corticosterone response at CT2, notwithstanding the blockade of a VPergic inhibition, is caused by the simultaneous lack of a stimulatory input to the CRH neuron during the early morning. The decreasing levels of corticosterone during the dark period, in spite of the absence of an inhibitory VP signal, are explained by the concomitant decline of the stimu-

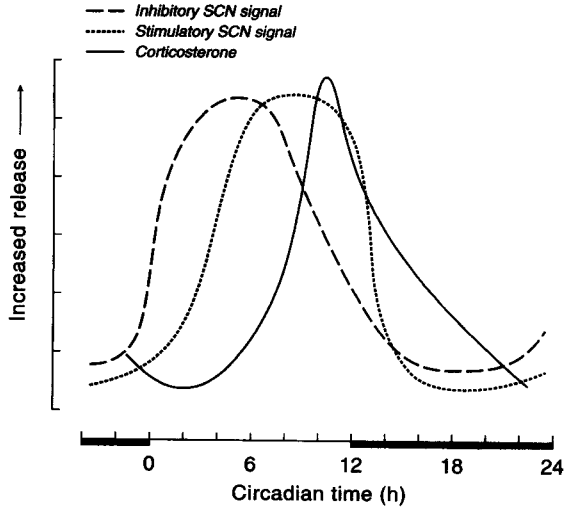

Fig. 8. Schematic presentation of the diurnal release pattern of SCN transmitters involved in the circadian control of corticosterone release.

latory input to the HPA system. Together, both rhythmic inputs from the SCN (i.e. the inhibitory and the stimulatory one) in the presently proposed phase relation (Fig. 8) are able to explain fully the observed rhythm in plasma corticosterone. The shape and height of the endogenous corticosterone peak is then defined by the internal synchronization of both rhythms. The importance of this 'tuning' of the different circadian rhythms is indicated by the study of Sparrow et al. (1993). This prospective study in humans shows that the height of the daily corticosterone peak is inversely related to the annual rate of decline of pulmonary function with aging (Sparrow et al., 1993). Furthermore, a broadening of the diurnal corticosterone peak in aged rats correlates with enhanced cognitive impairments (Issa et al., 1990).

In previous years, the apparent intact circadian rhythmicity of the VP-deficient Brattleboro rat has frequently been put forward as evidence against the notion that VP serves as an output signal of the circadian timing system (Ikonomov and Stoynev, 1994). The above scenario for the circadian control of basal corticosterone release explains why the Brattleboro rat may still show circadian behavior in general, and a circadian pattern of corticosterone release in particular. Due to the diurnal fluctuation of its stimulatory input to the HPA-axis, the SCN will still be able to create a circadian pattern of corticosterone release. However, without the inhibitory signal of VP, the timing of the peak will be different, viz. the onset of its daily rise will be earlier as compared to heterozygous animals. In fact, this premature rise of corticosterone in the Brattleboro rat was already observed by Ixart et al. (1982).

SCN–adrenal cortex interaction

In a further series of experiments, intact and SCN-lesioned animals were exposed to a novel environment stress. Their hormonal responses with regard to ACTH and corticosterone were followed (Buijs et al., 1997).

Placing intact animals in a new environment results in different plasma corticosterone and ACTH response, depending on the clock time of the stimulus. (1) Novel environment (2 h after onset of darkness (ZT14) results in a fast decrease followed by an increase in corticosterone. This changing pattern in corticosterone secretion was not accompanied by any change in plasma ACTH, suggesting a direct neuronal control of the adrenal cortex. (2) In contrast, novel environment at 2 h after light onset (ZT2) results in a rapid increase in plasma ACTH. Regression analysis of the relation ACTH-corticosterone before and after stress shows a changed pattern at ZT2, although at that time still no significant correlation between ACTH and corticosterone was detected. At ZT14 this correlation was only present after stress. (3) SCN lesioning results in low basal ACTH at all circadian times combined with elevated corticosterone levels. Here, a new environment results in an immediate increase in corticosterone without inhibition; ACTH also increases rapidly, but attains lower levels than at ZT2 in intact animals. These results therefore demonstrate SCN modulating corticosterone secretion by affecting ACTH secretion and changing the sensitivity of the adrenal cortex by means of a neuronal input (Buijs et al., 1997).

A direct inhibitory influence of the CNS on corticosterone secretion was reported by Jasper and Engeland (1994), who demonstrated that corticosterone secretion from the adrenal is increased during the diurnal trough after the splanchnic nerve is cut. Consequently, these observations provide physiological evidence for a (multisynaptic) SCN-adrenal cortex pathway comparable to the SCN-pineal pathway (Fig. 9).

We also propose that in addition to the SCN pathway, which increases ACTH at the end of the inactive period, a second SCN pathway should be present, able to interfere with ascending or descending stress input to the PVN. Such a connection should suppress this stress input to the HPA axis at ZT14 and enhance it at ZT2. These observations argue for an elaborate control mechanism regulating the circadian pattern in ACTH and corticosterone secretion and the way the levels of these hormones respond to stress. We propose that this control mechanism consists of at least three components.

1. An excitatory input from the SCN to (the dendrites of) the CRH neurons (Hermes et al., 1996) for the

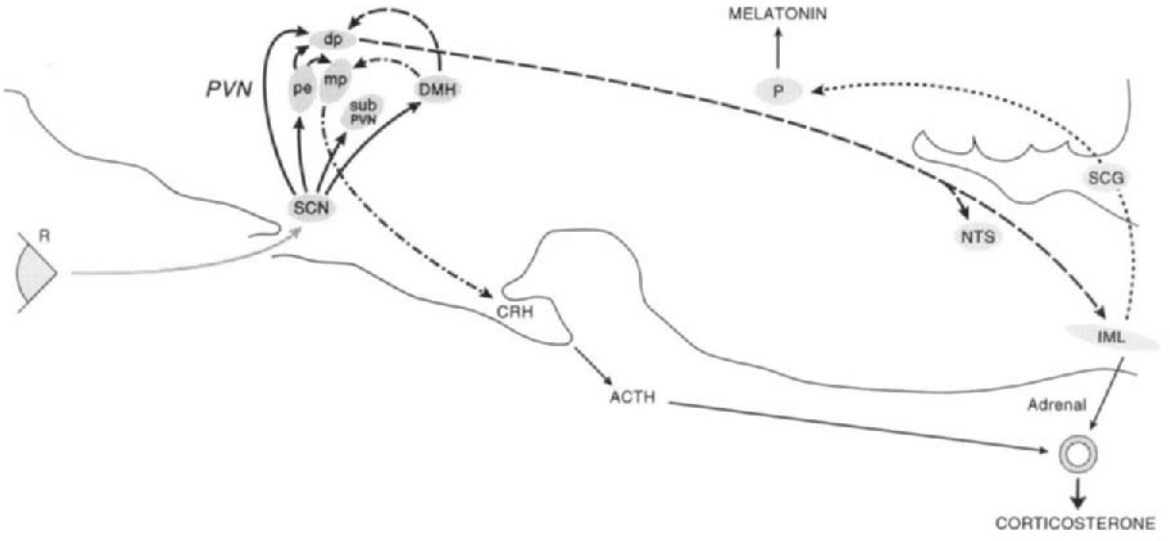

Fig. 9. Illustration of a sagittal scheme of the rat hypothalamus with the projection of the SCN to the DMH with the main projections of the DMH. Under the scheme is indicated which functions might be influenced by those projections. VMH, ventromedial hypothalamus; PVNmp, PVN medial parvocellular part; PVNdp, PVN dorsal part; IML, intermediolateral column; SCG, superior cervical ganglion; P, pineal; NTS, nucleus tractus solitarius; for other abbreviations see Fig. 1 legend.

circadian setting of the basal ACTH level, which can be activated by novelty stress only at ZT2.

2. An input from the SCN to spinal cord-projecting neurons in the PVN for the circadian setting of the responsiveness of the adrenal cortex. This pathway is also activated by novelty stress. This input results in an inhibition of corticosterone secretion. Both the CRH and the spinal cord-projecting neurons can also be indirectly activated by an input from neurons around the PVN.
3. SCN inputs to neurons receiving a stress input in and around the PVN which serves to modulate the stress input to PVN neurons.

The balance obtained by this system is not only influenced by stress, but also by many other environmental and endogenous factors, such as water and food intake, light, time of the day, and glucocorticoid feedback. The present study demonstrates that the SCN is one of the structures through which these factors may reach their effect. It also puts the SCN in the position of integrator between the periphery and the CNS, adding the time of the day information to this incoming peripheral information. Together with its multisynaptic pathway to the adrenal cortex, this integrative function of the SCN may form the basis for an explanation of phenomena, such as discrepancies in ACTH and cortisol levels and failing dexamethasone suppression tests during mental depression and Alzheimer's disease (Linkowski et al., 1985; O'Brien et al., 1996), of which in the latter disease a clear-cut deterioration of the SCN is present (Swaab et al., 1985).

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 4.4

Arginine vasopressin, fever and temperature regulation

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While central administration of arginine vasopressin (VP) to the non-febrile rat at high doses can cause hypothermia, there is little evidence for a role for endogenous VP in normal thermoregulation. In contrast, VP arising from cell bodies in the bed nucleus of the stria terminalis and innervating the ventral septal areas and possibly the amygdala appears to be an endogenous antipyretic, i.e. a substance capable of reducing fever. As the synthesis of VP in bed nucleus neurons is dependent upon circulating androgens, female rats have much less VP in these cells and their projections than do male rats. In keeping with this, females may make use of VP to a lessor extent than do males to bring about antipyresis. The phenomenon whereby the VP receptor can become sensitized by previous exposure to VP may be responsible for some states of endogenous antipyresis, in which fevers are suppressed through overactivity of the vasopressinergic system. States of endogenous antipyresis can be revealed around the time of parturition in both the neonate and the mother.

Introduction

The means by which neurons communicate with each other is through release of neurotransmitters. Many substances have been implicated as neurotransmitters involved in central thermoregulatory pathways (Pittman and Thornhill, 1990). In this review we will examine some of the evidence that the neuropeptide, arginine vasopressin (VP), is a neurotransmitter involved in central control of body temperature during normal thermoregulation and fever.

VP in normal thermoregulation

The possible participation of VP in thermoregulation was first suggested some 60 years ago. The neurosurgeon, Cushing (1931), injected an extract of the posterior pituitary gland (pituitrin) into the ventricles of a patient and observed sweating, vasodilatation and hypothermia. At the time, the active ingredient of this extract was not yet known, and it is likely that there were many other substances in addition to VP (at that time not yet characterized). Subsequently, Okuno and colleagues injected high doses of VP into the carotid artery of hyperthermic rats and observed a reduction in temperature (Okuno et al., 1965). More recently a number of studies have reported that central administration of high doses of VP into the lateral ventricle (intracerebroventricular; i.c.v.) or brain tissue of rats also causes hypothermia (Meisenberg and Simmons, 1984; Naylor et al., 1986; De Wied et al., 1993; Diamant and De Wied, 1993; Drago et al., 1997). Both structure-activity relationships and blockade by antagonists indicate the involvement of the V_1 type receptor. While these studies revealed a reduction in normal body temperature, a recent study reported that i.c.v. VP in nanogram quantities was able to reduce emotional hyperthermia resulting from restraint stress (Terlouw et al., 1996). This low dose contrasts with the microgram doses that are often necessary to elicit hypothermia at normal body temperatures and may reveal a preferential

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action of VP upon pathways activated at higher body temperatures. Hyperthermia can also be elicted in rats by cooling of the preoptic area, which results in a vigorous metabolic response. In such animals, infusion of VP into the lateral septum completely suppresses the metabolic response and hyperthermia (Banet and Wieland, 1985). There have been few attempts to uncover a role for endogenous VP in normal thermoregulation. One notable study however, has revealed an action of the V_1 antagonist, given i.c.v. in attenuation of restraint stress-induced hypothermia (Chen and Herbert, 1995).

While the above studies all report action of VP in the rat, it is possible that its action is quite different in other species. I.c.v. VP apparently has little or no effect upon normal thermoregulation in monkeys (Lee et al., 1985) or rabbits (Bernardini et al., 1983; Naylor et al., 1985b). Most notable are the observations that in the European hamster, VP could both interfere with hibernation (and its associated hypothermia) as well as cause arousal from hibernation (Hermes et al., 1989, 1993). In these animals, vasopressin immunoreactivity in the lateral septum shows a seasonal variation, being present during summer and absent in autumn and winter (Buijs et al., 1986). While these studies are consistent with an action of VP within thermoregulatory pathways, it is also possible that they reflect a primary action upon arousal, with the associated temperature changes not necessarily related to VP.

While there is a limited, possibly unphysiological (especially in rats) action of VP upon normal thermoregulation, there is now an overwhelming body of evidence supporting a role for VP in the control of body temperature in the febrile state. Before discussing in more detail the actions of VP in this regard, it may be appropriate to briefly review current concepts concerning the development of fever.

Fever

To understand antipyresis, it is important to know the basics of the cascade of events underlying the fever response. Several recent reviews cover this field in depth (Kluger, 1991; Moltz, 1993; Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995; Elmquist et al., 1997), but the following provides an overview. When microorganisms (gram negative infections are best understood) invade our body, they expose our immune system to large lipopolysaccharide molecules (LPS) often called exogenous pyrogens or endotoxins. LPS binds to a soluble, circulating LPS binding protein and this complex binds to the CD14 surface receptor found on certain monocytes and macrophages. These in turn synthesize and release a variety of endogenous peptides; those thought to be most important in fever are interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNFα). During fever, a number of humoral changes take place, collectively called the acute phase reponse. While fever is a hallmark of this response, in many cases the febrile response is preceded by an hypothermic period, the mechanism of which is not well understood. The regulated rise in body temperature characteristic of fever involves the CNS and the mechanism by which peripherally generated cytokines or other peptides activate the brain has been hotly debated. Evidence exists for several possible avenues, depending upon the route and dose of administration of cytokines (reviewed in Blatteis and Sehic, 1997a; Elmquist et al., 1997); these include direct transport across the blood brain barrier, entry at circumventricular organs, local stimulation of perivascular and meningeal cells, and activation of peripheral nerves. Whatever the route of administration, it appears that most cytokines activate an inducible cyclooxygenase (COX 2), most likely in glia, to cause central synthesis of prostaglandins, largely of the E series (PGE; reviewed in Blatteis and Sehic, 1997b; Elmquist et al., 1997). Peripheral immune stimuli activate many autonomic and endocrine nuclei, as revealed by Fos expression (Wan et al., 1994; Rivest and Laflamme, 1995a; Elmquist et al., 1996), but it is difficult to distinguish which pathways are involved in the fever response and which are involved in the many other autonomic responses (cardovascular, gastrointestinal, etc.) associated with immune activation, especially at the high doses often employed in these studies. Prostaglandins are known to act in several sites to activate central sympathetic pathways (reviewed in Blatteis and Sehic, 1997b), but the most sensitive of these for the purposes of fever

generation appears to be a small nucleus in the ventral medial preoptic area (VMPOA) (Scammell et al., 1996; Elmquist et al., 1997). Among other projections of this nucleus, that to the paraventricular nucleus (PVN) and nearby perifornical area appear to be particularly important sites for activation of heat conservation and thermogenesis to cause fever. In addition to this prostaglandin link, intense (i.e. high dose) peripheral immune activation causes synthesis within the brain of a variety of cytokines and certain transcription factors (Hopkins and Rothwell, 1995). While application of IL-1 or TNF directly to the brain by i.c.v. injection will cause a fever, and receptors of such cytokines are present in the brain, the involvement of this brain cytokine system in the responses to peripheral immune stimuli are not well understood. Nonetheless, for some models of fever, particularly those with long latencies, injection of IL-1 receptor antagonist (IL-1ra) into the brain will inhibit fever due to peripheral inflammation (Luheshi et al., 1993, 1996).

VP as an antipyretic

A possible role for VP as an antipyretic was prompted by observations that newborn lambs were very unresponsive to pyrogens, yet were well able to respond to a cold stimulus with heat production and limitation of heat loss (Pittman et al., 1973). Subsequently it was found that at the time of parturition, the ewes were also hyporesponsive to pyrogen induced fever (reviewed in Cooper et al., 1979a,b; Lederis et al., 1982). In searching for a possible agent which could induce this state of antipyresis, Kasting and colleagues found that VP, microinfused into the ventral septal area (VSA) of the sheep specifically reduced fever, but not normal body temperature (Cooper et al., 1979a,b). Many studies since that time have appeared describing similar effects of VP in other animals (reviewed in Moltz, 1993); as the evidence is most persuasive for the rat and the guinea pig, most of the following review will refer to studies in these animals.

Antipyresis after exogenous VP administration

In most mammals tested, injections or infusion of VP into the VSA are effective in lowering fever. Relatively few other loci have been systematically surveyed for sensitivity to VP's antipyretic effects; of those that have, the lateral septum (Naylor et al., 1985b) and the fundus striatum (Kremarik et al., 1995) are unresponsive, even though they contain VP receptors. On the other hand, in the central medial amygdala, microinjection of VP reduces PGE fever (Federico et al., 1992). This profile of sensitivity is not necessarily identical in all mammals; in guinea pigs it appears to be the lateral septum that is the antipyretic locus (Zeisberger, 1985; Roth and Zeisberger, 1992).

Pharmacology of VP action

Actions of VP are dose-dependent, can be blocked with V_1 antagonists and the effects are not mimicked by equimolar doses of less active analogs (Kovács et al., 1992). These observations indicate receptor-mediated actions via a V_1 type receptor, the major receptor type found in the brain (Ostrowski et al., 1992; Szot et al., 1994). In keeping with this possibility, VP receptors of the V_1 type are found in the VSA (Poulin et al., 1988; Johnson et al., 1993; Barberis and Tribollet, 1996).

The pharmacology of brain VP receptors is somewhat peculiar in that VP appears to be able to sensitize its own action in the brain. That is, upon repetitive exposure at the right interval and concentration, a number of the responses to the peptide are enhanced. This occurs without any change in receptor number or density, but through an amplification of the intracellular messengers, in particular those involving hydrolysis of phosphoinositol (Lebrun et al., 1990; Poulin and Pittman, 1993a). Another aspect of this peculiar sensitization is that the pharmacology of the sensitization is less dependent upon the V_1 receptor (Poulin et al., 1995). For example, recently we have discovered that pretreatment of rats with i.c.v. oxytocin, 24 h before induction of a fever, both attenuated the subsequent fever and also shifted the dose-response curve for VP such that lower doses were effective (Poulin and Pittman, 1993b). Yet, oxytocin itself is ineffective as an antipyretic. While the mechanism for this peculiar interaction is not yet understood, it

raises the possibility that states in which central oxytocin is released could affect fever.

Evidence for endogenous release of VP during fever

If VP is released endogenously during fever to bring body temperature back to normal, interference with the release or action of VP ought to result in elevated fever. We are fortunate in the fact that antagonists to the VP receptors are available and, following introduction of V_1 type antagonists, both experimental (Cooper et al., 1987) and naturally occurring (Cridland and Kasting, 1992) fevers are elevated. Likewise, if the VSA neurons, presumably carrying VP receptors, are destroyed by pretreatment with kainic acid, fevers are prolonged (Martin et al., 1988). Another way in which the endogenous VP innervation can be interfered with is through inactivation of VP synthesis in the cell bodies in the bed nucleus of the stria terminalis (BST). This can be accomplished by withdrawal of sex steroids by castration, as the VP synthesis in the BST is dependent on such steroids (DeVries et al., 1985); when experimental fevers are initiated in such castrated animals, fevers are augmented (Pittman et al., 1988). Castrated animals also show an attenuation of the initial hypothermic response after LPS which precedes the development of fever (Derijk and Berkenbosch, 1994).

If inactivation of BST neurons is associated with an elevation of fever, it follows that their activation should reduce fever. In keeping with this, when BST neurons are activated by electrical stimulation, fevers are suppressed and this suppression can be reversed if the VSA is pretreated with a VP antagonist (Naylor et al., 1988). These neurons are also activated during fever initiated by i.c.v. PGE1, as a small number of the neurons in the BST which project to the VSA show activity patterns consistent with those predicted for vasopressinergic neurons responsible for antipyresis (Mathieson et al., 1989). This population of neurons in the BST also displays Fos immunoreactivity after LPS, which is in particular associated with the hypothermia that sometimes accompanies LPS administration (Hare et al., 1995). If we carry out dialysis of the VSA, and measure VP release during fever, we can detect increased release (Landgraf et al., 1990; Chen et al., 1997), a phenomenon first reported by Kasting many years ago in sheep (Cooper et al., 1979).

Female rats naturally have lower levels of circulating androgens, leading to reduced synthesis of VP in the BST-VSA pathway. This leads to a striking sexual dimorphism of the vasopressinergic innervation and caused us to examine febrile responses in female rats and compare them with those seen in male rats. In both conscious and in anesthetized rats, we have observed that female rats had higher PGE_2 fevers than did males (Chen et al., 1997). Such a finding is consistent with the reduced VP innervation of the VSA as the cause of this enhanced fever, and in keeping with this, PGE_2 fevers in females are neither associated with enhanced release of VP in the VSA, nor are they elevated by the VP antagonist. These observations raise the possibility that females may not depend upon VP as an antipyretic to the same extent as do male rats.

Natural stimuli for VP release

During a fever, animals may be exposed to a variety of pyrogens, the body temperature is elevated and there are often a number of changes in other regulatory functions (e.g. blood pressure). Any or all of these could provide the stimulus for activation of the central vasopressinergic antipyretic pathways. The dialysis studies referred to above indicate that PGE itself can cause VP release in the VSA, at least when body temperature is concurrently elevated (it seems as if an elevation in body temperature alone is insufficient to activate VP release). Nonetheless, the fevers due to PGE represent only one aspect of the response to peripheral LPS, and it is likely that the central nervous system changes to LPS, or indeed to infection, are more complex than that produced by PGE alone. For example, there is now evidence that there may be additional synthesis in glial cells of interleukins in response to peripheral LPS (Van Dam et al., 1995). This appears to be a possible stimulus for the release of VP. We showed that IL-1β, applied iontophoretically or by micropressure to BST neurons, caused increased electrical activity (Wilk-

inson et al., 1993). A subsequent experiment indicated that at least some of such cells were vasopressinergic in nature. When push-pull perfusions of the VSA were carried out and IL-1β was microinfused into the BST, there was a dose-dependent rise in the quantity of immunoreactive VP in the perfusates (Wilkinson et al., 1994). Thus, it is possible that cytokines both initiate the febrile process and at the same time set in motion the mechanisms to limit their action.

The peripheral changes that accompany LPS administration could also activate the central antipyretic system. For example, at higher doses often employed in experimental studies, significant hypotension can result from peripheral LPS; as central VP pathways can also be activated by hypotension (Petrov et al., 1995; Krukoff et al., 1997) and hypotension itself can reduce LPS fever (Kasting, 1996), peripheral cardiovascular changes could account for a portion of the VP-induced antipyresis. In support of this are observations that BST neurons are activated by cardiovascular stimuli (Wilkinson and Pittman, 1995).

There is some evidence that peripheral generation of cytokines plays a role in the activation of the central VP system. In particular, it has been reported that the hypothermic response that often accompanies LPS administration is associated with elaboration of circulating TNFα, which in turn activates the VSA VP system (Derijk and Berkenbosch, 1994). Indeed TNFα has been proposed as an antipyretic agent (Long et al., 1990).

Circulating VP – Does it have a role in antipyresis?

Current dogma is that circulating VP does not affect fever, based upon experiments in which peripheral administration of VP was without effect on fever (see Kasting, 1989 for review). However, as LPS administration activates vasopressinergic magnocellular neurons in the hypothalamus (Ericsson et al., 1994; Rivest et al., 1995b) and fever is associated with high circulating levels of VP (Kasting et al., 1985), receptors are most likely saturated and it would appear unlikely that exogenously applied VP would have much effect. Given that VP, released from the median eminence, potentiates corticotrophin releasing factor action on the pituitary–adrenal axis (Liu, 1994), and that corticosterone suppresses fever (Goujon et al., 1995a,b), it is possible that the plasma VP does have an antipyretic action. Another possibility is a direct action on the adrenal cortex to enhance corticosterone secretion. V_1 receptors have been localized to the adrenal (Balla et al., 1985) and VP increases corticosterone secretion in the isolated perfused adrenal gland (Hinson et al., 1987). If circulating VP were to be shown to promote defervescence, this would provide a role for pituitary VP complementing that of the centrally-released peptide.

Endogenous antipyresis and central VP

The above discussion dealt with possible roles for VP in promoting defervescence after fever, or indeed in limiting the height and magnitude of the febrile response. There are also a number of conditions in which fever responses are greatly attenuated, and VP has also been implicated in these states of 'endogenous antipyresis' (reviewed in Pittman and Wilkinson, 1992).

In the newborns of a number of species, including sheep (Pittman et al., 1973; Cooper et al., 1979), guinea pigs (Blatteis, 1975) and rabbits (Szekely, 1978), there is little or no febrile response to LPS in the immediate few hours or days after birth. This suppression occurred despite the capability to mount a thermogenic response. A similar suppression was seen in neonatal rats, but when such animals were pretreated with a VP antagonist, they were able to develop a fever through behavioral means after LPS (Kasting and Wilkinson, 1987). These studies indicated that endogenous VP was suppressing fever.

A similar suppression occurs during pregnancy, parturition and lactation in several species (Kasting et al., 1978; Zeisberger et al., 1981). In rats given PGE i.c.v., we found a significant depression in the maximal change in body temperature as well as an enhanced defervescence during lactation, around the time of parturition and over the first few days of lactation (Martin et al., 1996). A similar depression of the febrile response to intravenously injected LPS was also seen at these times which

was furthermore associated with an absolute suppression of fever around the time of parturition (Martin et al., 1995). The observations of these reduced fevers raise the possibility that an endogenous antipyretic system may be activated in these rats during these phases of the reproductive cycle. It may be of interest that enhanced release of VP occurs in the VSA during these times (Landgraf et al., 1991). While there is still no firm evidence that the VP system is involved, in guinea pigs at parturition, VP immunoreactivity is markedly increased (Zeisberger et al., 1981; Roth and Zeisberger, 1992). The involvement of VP in fever suppression in the rat is not yet known; it is possible that the reduced fevers are but one manifestation of a general suppression of sympathetic activity at this time.

VP and pathology

In addition to its antipyretic actions, VP is also known to cause severe motor disturbances in response to i.c.v. application (Kasting et al., 1980) or direct injection into the VSA (Naylor et al., 1985a) or amygdala (Willcox et al., 1992). It was originally proposed by Kasting and colleagues (Kasting et al., 1981) that VP could be a mediator of febrile convulsions, but there is still no direct evidence for this. It is interesting that these convulsive actions also display a sensitization to VP, in that they are more severe upon a second exposure within hours to days. We were able to reveal a possible interaction between fever and VP-induced convulsive activity by testing the convulsive response to VP in animals that had been given experimental fevers a day earlier. Such animals developed significantly more severe convulsions than controls that remained afebrile (Poulin and Pittman, 1993c). While these findings are provocative, there has still been no demonstration of VP-mediated convulsions in animals displaying sustained fevers. Further investigation is required in young animals, for febrile convulsions are a phenomenon of children, not adults.

Antipyretics such as aspirin and indomethacin, although better known as inhibitors of PGE synthesis, have also been shown to cause VP release in the VSA (Alexander et al., 1989; Wilkinson and Kasting, 1989). We have found that administration of salicylate or indomethacin to rats made tolerant to LPS causes hypothermia and motor disturbances and even death at doses which cause only antipyresis in the non-tolerant rats (Wilkinson and Pittman, 1994). As there is some evidence that tolerance to LPS might involve central VP receptors (Kasting and Wilkinson, 1987; Cooper et al., 1988), we have suggested that previous release of VP in response to LPS sensitized the VP receptors. When the antipyretics were administered, the VP they caused to be released abnormally activated these receptors. There are some similarities between the responses that we have described and the responses of some children who take aspirin after a prodromal viral illness (Reyes syndrome).

Conclusion

There is now a great deal of evidence that VP may function as an antipyretic, yet animals entirely lacking VP are able to defervesce after a fever. There are obviously other endogenous substances that are capable of acting as antipyretics (Huang et al., 1997), and it will be important to determine how the various antipyretics interact. In particular, it will be important to determine if female rats make use of the VP antipyretic system during fever and at times when fever is suppressed. Future studies must also determine exactly how the VP acts, at the cellular level, to cause antipyresis.

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SECTION 5

Vasopressin and integrated brain functions

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 5.1

Role of vasopressin and oxytocin in the control of social behavior in Syrian hamsters (*Mesocricetus auratus*)

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Vasopressin (VP) and oxytocin (OT) play an important role in regulating social behavior in a variety of species as a result of their actions in the central nervous system. The following paper reviews the actions of VP and OT in controlling a range of social behaviors involved in communication, aggression and reproduction in the Syrian hamster. These data suggest that social and hormonal stimuli alter the expression of specific social behaviors by altering the release of, or the response to, VP and OT within key elements of the neural circuits controlling these behaviors.

Introduction

There is a considerable body of evidence that neural circuits containing vasopressin (VP) and oxytocin (OT) within the CNS are involved in the regulation of social behavior in a variety of species. This paper discusses the role of VP and OT in controlling social behaviors in Syrian hamsters (*Mesocricetus auratus*). Syrian hamsters have proven to be an excellent model for these studies because they display dramatic and rapid alterations in a variety of social behaviors.

VP and hamster scent marking

Our original studies on the role of VP in social behavior began with the serendipitous finding that VP injected into the rostral hypothalamus stimulated a form of scent marking behavior called flank marking. Hamsters flank mark by rubbing a pigmented sebaceous gland located on their flank against objects in the environment (Johnston, 1985). Flank marking occurs in a variety of social situations and can be stimulated by the odors of other hamsters. This behavior is used to communicate several different types of social information such as dominance status (Johnston, 1975, 1977; Ferris et al., 1987) and mate choice (Huck et al., 1985). After the initial observation that VP stimulated flank marking (Ferris et al., 1984), we investigated whether VP is an essential component of the neural circuitry controlling this behavior (see Albers et al., 1992; Ferris, 1992, for reviews). These studies demonstrated that VP stimulated flank marking in a dose-dependent manner (Albers et al., 1986, 1991; Ferris et al., 1988) and that VP antagonists significantly inhibited flank marking stimulated by VP, the odors of other hamsters or social interactions (Ferris et al., 1985b, 1986b; Albers et al., 1986b). Furthermore, VP-stimulated flank marking was not found to require peripheral feedback from the flank gland since VP continued to stimulate flank marking in hamsters following removal of their flank gland (Albers and Ferris, 1986). Anatomical studies revealed that the VP-responsive region contains a significant number of VP immunoreactive (IR) fibers (Ferris et al., 1989; Dubois-Dauphin et al., 1990; Albers et al., 1991) and VP binding sites (Dubois-Dauphin et al., 1990; Ferris et al., 1993; Johnson et al., 1995). Structure-activity studies and data obtained with selective VP

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antagonists indicated that a V_{1a}-like VP receptor mediates the effects of VP on flank marking (Albers et al., 1986) (Fig. 1).

Neural circuitry controlling flank marking

One of the interesting features of VP's ability to stimulate flank marking is that the VP-responsive region extends in a relatively long zone from the posterior medial and lateral preoptic area (MPOA) to the posterior medial and lateral aspects of the anterior hypothalamus (AH) (referred to from here on as the MPOA-AH). This region was localized using unilateral injections of VP in a small volume of vehicle (10—100 nl) to minimize its spread (Fig. 2). Lesion studies indicated that destruction of a relatively small part of this zone was sufficient to significantly reduce flank marking (Ferris et al., 1986a). In addition, stimulation of flank marking by unilateral injection of VP was blocked by lesions of the contralateral anterior hypothalamus (Ferris et al., 1994). Taken together these data suggest that the expression of flank marking requires the recruitment of a relatively large number of neurons in the VP-responsive region of the MPOA-AH, however stimulation of a subpopulation of these neurons with VP can activate the circuitry controlling the behavior and initiate its expression.

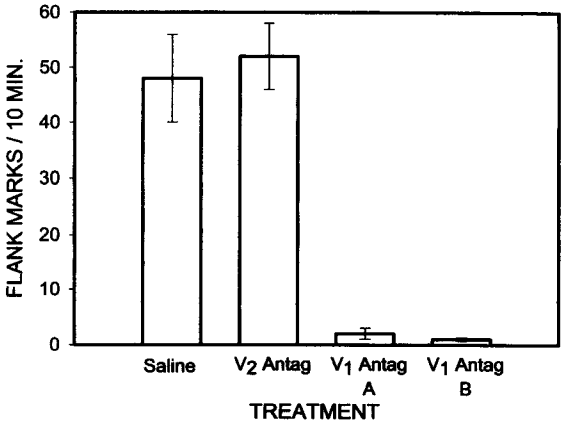

Fig. 1. Inhibition of VP-induced flank marking by injection of selective V_{1a} antagonists (V_1 Antag A: $d(CH_2)_5Tyr(Me)$-VP and V_1 Antag B: dPTyr(Me)VP), but not by injection of saline or a V_2 antagonist (V_2 Antag: $d(CH_2)_5(D\text{-}Ile^2,Ile^4)VP$ the MPOA-AH. From Albers et al., 1986; used with permission from the *Journal of Neuroscience*.

The VP-responsive region of the MPOA-AH contains significant numbers of VP-IR fibers and receives afferent input from a variety of CNS sites (Albers et al., 1992). The location of the neurons that regulate the expression of flank marking by releasing VP within the VP-responsive region have not been defined. The distribution, projections and regulation of VP-IR neurons in hamsters differs in a number of ways from that of other rodents and even other species of hamsters (Buijs et al., 1986; De Vries, 1990; Bittman et al., 1991; Bamshad et al., 1993). For example, several populations of parvocellular neurons that express VP-IR in rats are absent in Syrian hamsters (Albers et al., 1991; Ferris et al., 1995). The magnocellular VP system of hamsters also differs in important ways from that of rats. Unlike rats, VP-IR magnocellular neurons in hamsters do not project exclusively to the posterior pituitary, (Mahoney et al., 1990; Ferris et al., 1991) so it is possible that VP magnocellular neurons could be involved in the control of flank marking. Destruction of the magnocellular VP neurons that form the hypothalmo–neurohypophysial system produces the expected decreases in plasma VP levels and the resultant increases in water intake and urine output but does not eliminate the expression of flank marking. It has been estimated that 30% of the neurons in and around the VP-responsive region of the MPOA-AH do not project to the neurohypophysis. The VP-IR neurons that do not project to the neurohypophysis are found primarily in parts of the supraoptic nuclei, paraventricular nucleus and nucleus circularis. It has been proposed that at least some of the VP-IR cell bodies important in the regulation of flank marking are in the supraoptic nucleus. Unilateral kainic acid lesions of neurons in the supraoptic nucleus significantly reduce flank marking stimulated by the odors of other hamsters (Ferris et al., 1990b). It appears that these lesions disrupt the input to the VP-responsive region and not its efferent system since they do not eliminate the ability of VP to stimulate flank marking when

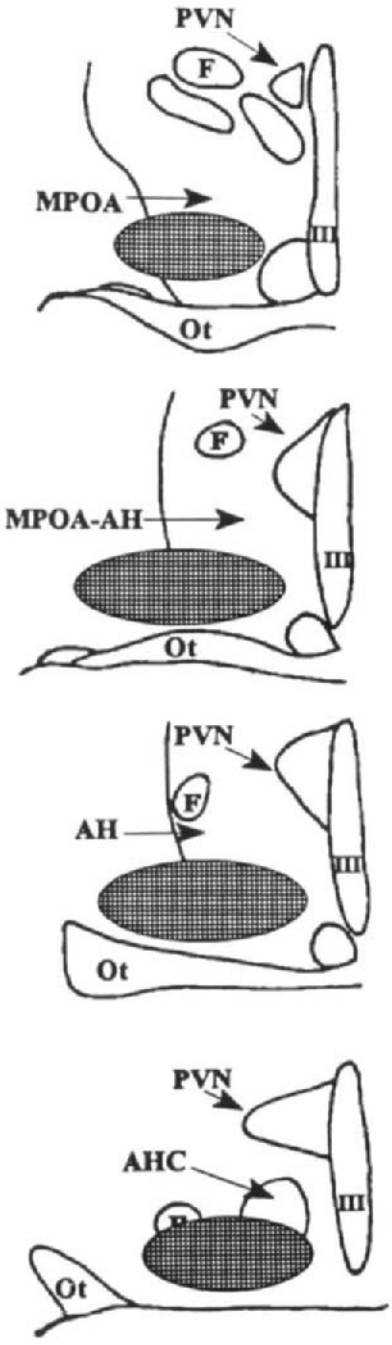

Fig. 2. Approximate boundaries of the zone within the MPOA-AH where small volume injections of VP (10–100 nl) stimulate flank marking. PVN, paraventricular nucleus; F, fornix; MPOA, medial preoptic area; III, third ventricle; Ot, optic chiasm; AH, anterior hypothalamus.

injected into the MPOA-AH. In contrast, lesions of neurons in the paraventricular nucleus do not significantly reduce odor-stimulated flank marking or marking induced by the injection of VP into the MPOA-AH. Other recent studies employing double-labeling techniques also indicate that neurons in the supraoptic nucleus are important in flank marking (Bamshad and Albers, 1996). More neurons in the supraoptic nucleus that project to the VP-responsive region of the MPOA-AH exhibit Fos-IR after induction of flank marking by VP than after control injections of saline. While these data support the overall hypothesis that neurons in the supraoptic nucleus that project to the VP-responsive region are important in the control of flank marking it is not clear why they are activated in response to flank marking stimulated by VP injected into the MPOA-AH. More data will be required to define whether projections from the supraoptic nucleus release VP into the VP-responsive region and thereby stimulate flank marking as part of the normal control of the behavior.

Neuroanatomical studies have also shown that the VP-responsive region in the MPOA-AH is connected to several other sites that appear to be involved in the regulation of flank marking by VP. For example, neuroanatomical tracing studies have shown that the VP-responsive region in the MPOA-AH is reciprocally connected with the lateral septum (LS), the bed nucleus of the stria terminalis (BNST) and the periaqueductal gray (PAG) (Ferris et al., 1990a; Albers et al., 1992a). VP receptor binding is found in the LS, BNST and PAG (Ferris et al., 1993; Johnson et al., 1995), and microinjection of VP into these regions stimulates flank marking (Irvin et al., 1990; Hennessey et al., 1992). Additional support for a role of these sites in the control of flank marking comes from studies employing Fos-IR (Bamshad and Albers, 1996). Stimulation of flank marking by the injection of VP into the VP-responsive region of the MPOA-AH increases the number of cells expressing Fos-IR in the BNST and PAG over that seen in saline-injected controls. An unexpected finding was that higher levels of Fos-IR were found in the central nucleus of the amygdala (Ce) in hamsters that flank marked in response to VP. The possible role of the

Ce in flank marking has been supported further by the finding that lesions of the Ce reduce flank marking stimulated by VP injected into the VP-responsive region of the MPOA-AH (Bamshad et al., 1996b). It is interesting, however, that although substantial levels of VP receptor binding exists in the Ce (Ferris et al., 1993; Johnson et al., 1995), VP injected into this region does not stimulate flank marking (Bamshad et al., 1996b). In summary, it appears that several of the sites involved in regulation of flank marking are anatomically interconnected and may represent components of the neural circuit controlling the behavior.

VP and aggression

In addition to flank marking, VP appears to play an important role in regulating aggression in hamsters. Injection of a selective V_{1a} VP receptor antagonist into the AH causes a dose-dependent reduction in offensive aggression by a resident male hamster toward a male intruder (Ferris and Potegal, 1988). In addition, injection of a V_{1a} VP receptor antagonist also inhibits aggression between two male hamsters placed in a neutral arena (Potegal and Ferris, 1989). The region where VP influences aggression appears to at least partially overlap the zone where VP stimulates flank marking, however it is not known if the same neurons are involved in controlling both behaviors. It has been reported that concentrations of VP higher than 0.9 μM injected into the AH stimulate flank marking in resident hamsters even when intruders are placed in their home cage. However, injection of 0.09 μM VP into the AH, which typically produces lower levels of flank marking, increases the aggression of resident hamsters toward male intruders (Ferris et al., 1997). These data suggest the interesting hypothesis that the behavior stimulated by VP released within the AH depends on its concentration. At low concentrations, VP stimulates aggression and at higher concentrations VP stimulates flank marking.

Another site where VP may serve to regulate aggressive behavior is the ventrolateral hypothalamus (VLH). Injection of VP into this region facilitates offensive aggression in males (Delville et al., 1996). However, it will be important to determine whether inhibition of VP activity within the VLH inhibits the normal expression of aggression.

Aggressive behavior and flank marking play important roles in the establishment and maintenance of dominant/subordinate relationships in hamsters. When two hamsters who have not previously interacted are placed together in a neutral arena for short periods of time over a number of days one hamster will become dominant and the other subordinate. Initially, the hamsters exhibit high levels of aggressive behavior with the hamster that ultimately becomes dominant displaying more aggression (Ferris et al., 1987). As the dominance relationship forms, aggressive behavior declines and the levels of flank marking increase such that the dominant hamster flank marks significantly more than does the subordinate. Flanking marking appears to be involved in establishing dominant/subordinate relationships. When two naive hamsters are tested together and the flank glands of one or both hamsters are removed no clear dominant subordinate relationship is formed and the hamsters continue to display high levels of aggression. Injection of a V_{1a} VP receptor antagonist into hamsters that have not previously interacted prevents the formation of a dominant/subordinate relationship (Ferris, 1992). The inability to form such a relationship may be the result of the inhibition of aggressive behavior. In hamsters that have established a dominant/subordinate relationship, injection of VP and VP antagonists can significantly alter the levels of flank marking displayed by both the dominant and subordinate hamster (Ferris et al., 1986b). Injection of VP into the MPOA-AH of the subordinate hamster stimulates high levels of flank marking despite the presence of the dominant hamster. Injection of an VP antagonist into the dominant hamster significantly reduces flank marking despite the presence of the subordinate hamster. The effects of VP and VP antagonists appear to be primarily on flank marking and not on the dominant/subordinate relationship because injection of these substances does not reverse the basic dominant/subordinate relationship of these hamsters even when given over three consecutive days.

The development of dominant/subordinate relationships appears to alter specific components of

the hamster VP system (Ferris et al., 1989). Subordinate hamsters in well established dominant/subordinate relationships have lower levels of VP-IR in the AH, but not other regions, when compared to dominant hamsters or to socially isolated controls. Immunohistochemical analysis also indicate that there are fewer VP-IR fibers in the AH and lower numbers of VP-IR cell bodies in the nucleus circularis in subordinates when compared to dominant hamsters or controls. The protocol used in this experiment compared dominant hamsters that had been castrated and treated with testosterone to submissive hamsters that had been castrated but not given testosterone in order to maximize the behavioural differences between the pairs. However, it was unlikely that the differences in VP-IR were the result of the difference in testosterone levels since no differences in VP-IR was observed in controls that had been given the same hormone treatments but not the opportunity to socially interact. These data raise the interesting possibility that the formation of dominant/subordinate relationships alter the amount of VP and that the differences in VP levels may account for at least some of the differences in the levels of flank marking and aggression seen between dominant and subordinate hamsters.

Gonadal hormones and the regulation of flank marking and aggression by VP

In most mammalian species testosterone has a major influence on the expression of many social behaviors including scent marking (Johnson, 1973; Hart, 1974). In male hamsters, odor-stimulated flank marking slowly, but significantly, declines following castration, and exogenously administered testosterone can restore precastration levels of the behavior (Johnston, 1981). The role of gonadal hormones in the control of scent marking in female mammals is less clear than in males (Yahr, 1983). However, in female hamsters, the 4-day rhythm of ovarian hormones induces a 4-day rhythm in the frequency of odor-stimulated flank marking (Johnston, 1977; Albers and Rowland, 1989). In ovariectomized hamsters, physiological levels of estradiol restore diestrous levels of flank marking, and progesterone given to estradiol-primed, ovariectomized hamsters reduces levels of flank marking to those seen on estrus in intact females.

There is considerable evidence that the MPOA-AH mediates at least some of the effects of gonadal hormones on a number of different behaviors. The finding that VP within the MPOA-AH is a critical neurochemical signal for flank marking has provided the opportunity to test the hypothesis that gonadal hormones influence flank marking by altering the signaling capacity of VP. Specifically, gonadal hormones could influence flank marking by altering the amount of VP released or by altering the sensitivity or response to VP. It might be anticipated that gonadal hormones influence flank marking by altering the amount of VP in neurons controlling flank marking because gonadal hormones have been shown to have dramatic effects on VP-IR in a number of limbic structures in other species (De Vries et al., 1985). However, in hamsters, gonadal hormones produce no detectable alterations in VP-IR in the MPOA-AH or in a variety of other structures likely involved in the control of flank marking (Albers et al., 1991; Huhman and Albers, 1993; Hennessey et al., 1994). While the possibility that gonadal hormones influence flank marking by altering VP release can not be excluded, these data provide little support for this hypothesis. Instead, gonadal hormones appear to influence flank marking by altering the sensitivity or response of the MPOA-AH to VP. In males, testosterone significantly increases the amount of flank marking induced by VP microinjected into the MPOA-AH (Albers et al., 1988) (Fig. 3). Similarly, estradiol significantly increases the amount of flank marking produced by VP injected into the MPOA-AH in female hamsters (Huhman and Albers, 1993). The amount of flank marking stimulated by VP injected into the MPOA-AH varies as a function of the estrous cycle (Albers et al., 1996). The lowest levels of VP-stimulated flank marking occur on the day of estrus. The alterations in response to VP over the estrous cycle appear to result from the changing levels of ovarian hormones over the estrous cycle. Estradiol administered to ovariectomized hamsters increases the amount of VP-stimulated flank marking and progesterone given to estradiol-treated hamsters reduces VP-induced marking. These data suggest the possibility that

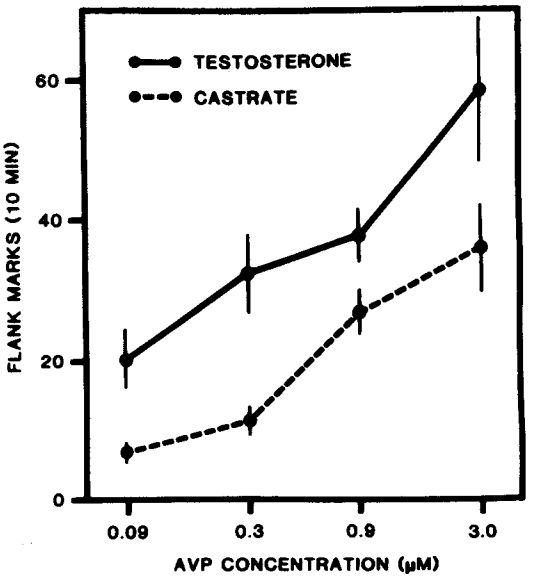

Fig. 3. Dose-dependent induction of flank marking by injection of VP into the MPOA-AH of castrated male hamsters (n = 18) or male hamsters with physiological levels of circulating testosterone (n = 17). The amount of VP-induced flank marking was significantly lower in the absence of testosterone. From Albers et al., 1988; used with permission from *Brain Research*.

gonadal hormones influence the amount of VP-induced flank marking in the MPOA-AH by altering VP binding. This hypothesis was supported by the finding that castration reduces V_{1a} receptor binding within the posterior lateral preoptic-anterior hypothalamic continuum (Johnson et al., 1995) (Fig. 4). In summary, these data indicate that the MPOA-AH is an important site in mediating the effects of gonadal hormones on flank marking and suggest that gonadal hormones may modulate flank marking by altering VP receptor binding at least in male hamsters.

It is not known if gonadal hormones influence behavior by acting in a parallel manner at multiple CNS sites or if gonadal hormones have a primary site of action in controlling specific behaviors. There is evidence that gonadal hormones act at multiple CNS sites to influence at least some behaviors, e.g. mating behavior in male hamsters (Wood and Newman, 1993). However, gonadal hormones may influence flank marking by acting primarily in the MPOA-AH (Albers and Cooper, 1995) since gonadal hormones have only small effects on the ability of VP to stimulate flank marking in the LS/BNST and PAG. At present, the simplest hypothesis is that the MPOA-AH is the primary site where gonadal hormones influence flank marking. Nevertheless, it seems unlikely that gonadal hormones act exclusively in the MPOA-AH to influence the behavior.

There is a considerable body of evidence that gonadal hormones influence aggression in a wide variety of species (Svare, 1983). In male hamsters androgens facilitate offensive aggression (Payne, 1973). The role of VP in controlling aggression in hamsters has only been investigated in males. It is not known whether testosterone influences the facilitation of aggression by VP injected into the AH. However, since castration reduces VP binding within this region (Johnson et al., 1995), it seems possible that castration could reduce VP-induced aggression. There is evidence that gonadal hormones influence the effects of VP on aggression

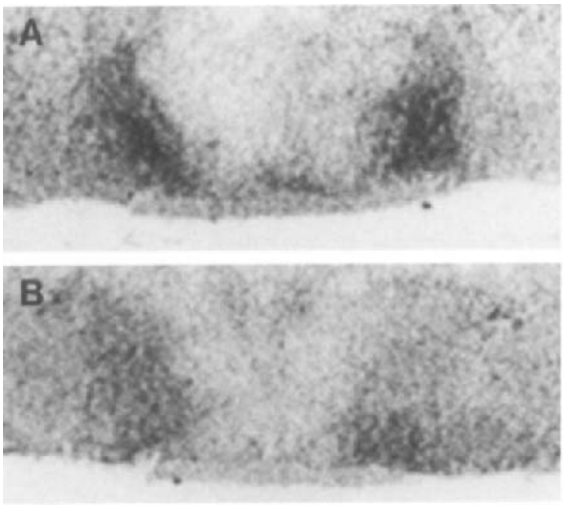

Fig. 4. Binding of a selective V_{1a} VP receptor ligand ([^{125}I]-linear VP antagonist in sham-castrated (A) and castrated male hamsters (B) at the level of the anterior hypothalamus. Note the significantly higher levels of binding in sham-castrates versus the castrates. Modified from Johnson et al., 1995.

within the VLH (Delville et al., 1996). VP injected into the VLH facilitates aggression in intact males and castrated males given testosterone but does not facilitate aggression in castrated controls. VP receptor binding is also reduced in the VLH following castration and pre-castration levels of binding can be restored by testosterone. Thus, the regulation of VP binding in the VLH may explain why VP is unable to stimulate aggression in castrated males when injected into this region.

Social context and the regulation of social behavior by VP

Social context is an important determinant of the type and amount of social behavior expressed by an individual. Little is known about the mechanisms that mediate the ability of social factors to influence the expression of specific social behaviors. One way in which social context might influence the expression of flank marking is by altering the response of the MPOA-AH to VP. One approach to examining this question is to determine whether the ability of VP to stimulate flank marking can be inhibited in a social situation where other behaviors are normally expressed at high levels. When female hamsters are in estrus and are placed in an arena with a male, the female exhibits high levels of sexual receptivity and little or no flank marking. To determine whether this social environment could inhibit the ability of VP to stimulate flank marking, VP was injected into the MPOA-AH of ovariectomized female hamsters given levels of estradiol and progesterone that induce high levels of sexual receptivity (Albers and Rawls, 1989). When the females were injected with VP and tested with a male, high levels of sexual receptivity occurred and little or no flank marking was observed. However, if these same females were injected with VP and tested alone, even immediately after the test with the male, VP stimulated high levels of flank marking. These data suggest that the ability of VP to stimulate flank marking in the MPOA-AH can be blocked by at least some social situations. However, the ability of VP to stimulate flank marking in the MPOA-AH is not blocked in all social situations where flank marking normally occurs at low levels. For example, despite the low levels of flank marking normally observed in subordinate hamsters, VP can still stimulate high levels of flank marking in the subordinate male even during encounters with their dominant partner (Ferris et al., 1986b).

VP interactions with other neurotransmitters

It seems likely that VP interacts with other neurochemical signals within the MPOA-AH to regulate the expression of flank marking. Glutamate is an excitatory neurotransmitter that appears to mediate fast synaptic neurotransmission in the hypothalamus (van den Pol and Trombley, 1993). To investigate whether VP interacts with excitatory amino acid neurotransmitters to regulate flank marking, VP was injected in combination with either NMDA or non-NMDA antagonists (Bamshad et al., 1996a). Both NMDA and non-NMDA glutamate antagonists were found to inhibit the ability of VP to stimulate flank marking in the MPOA-AH. Thus, excitatory amino acid neurotransmitters may interact with VP in regulating flank marking within the VP-responsive region of the MPOA-AH.

There is also evidence that two other neurotransmitters may serve to inhibit the behavioral effects of VP in the MPOA-AH. In female hamsters, injection of norepinephrine (NE) in combination with VP into the MPOA-AH produces a dose-dependent inhibition of VP-induced flank marking (Whitman et al., 1992) (Fig. 5). In contrast, the combined injection of either serotonin, dopamine or neuropeptide Y with VP does not diminish the levels of VP-induced flank marking. Injection of epinephrine (EPI) also has inhibitory effects on VP-induced flank marking in females although these effects are less consistent than those of NE. The similar effects of NE and EPI may be due to the ability of both NE and EPI to activate alpha and beta noradrenergic receptors. The VP-responsive region of the MPOA-AH contains fibers that exhibit tyrosine hydroxylase-IR and dopamine beta-hydroxylase-IR (Vincent, 1988) suggesting that NE could have a physiological role in altering the response of this region to VP. One very interesting feature of the

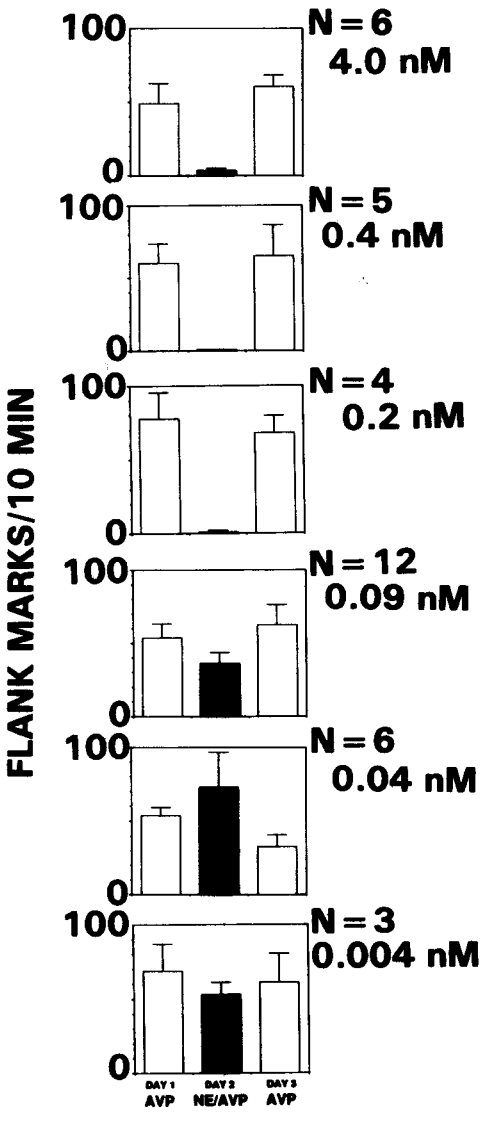

Fig. 5. Inhibition of VP-induced flank marking by NE. The open bars indicate the amount of flank marking observed following injection VP into the MPOA-AH. The filled bars indicate the amount of flank marking observed following injection of VP combined with one of six concentrations of NE into the MPOA-AH. A statistically significant inhibition of VP-induced marking was observed following injections of cocktails containing 4.0, 0.4 or 0.2 nM NE. Cocktails containing lower doses of NE did not significantly reduce VP-induced marking. From Whitman et al., 1992; used with permission from the *Journal of Neuroendocrinology*.

effects of NE is that it only inhibits VP-induced flank marking in female hamsters. In males, the combined injection of VP and NE produces no inhibition of VP-stimulated flank marking. Since VP facilitates aggression in at least some of the sites where it stimulates flank marking, it would seem possible that NE could inhibit VP-induced aggression. While there is no direct evidence on whether NE inhibits VP-induced aggression, NE does appear to inhibit at least some forms of aggression. Following the establishment of dominant/subordinate relationships in female hamsters, injection of NE into the MPOA-AH responsive region significantly reduces the numbers of attacks and bites displayed by dominant hamsters (Harmon et al., 1995). Given the prominent sex difference in the inhibitory effects of NE on VP, it would be interesting to determine whether sex differences exist in the inhibitory effects of NE on aggression in the MPOA-AH.

In male hamsters, serotonin (5-HT) within the AH and VLH appears to play an important role in modulating VP-induced social behavior. There is a dense field of VP-IR and 5-HT-IR fibers in the AH that are in the same region as VP and 5-HT binding sites in male hamsters (Ferris et al., 1997). VP-induced flank marking is inhibited when 5-HT is combined with VP and injected into the AH (Ferris and Delville, 1994). Similarly, the systemic administration of the 5-HT reuptake inhibitor, fluoxetine, inhibits flank marking stimulated by injection of VP into either the AH or VLH (Ferris and Delville, 1994). As discussed above, flank marking was not inhibited in female hamsters following the combined injection of VP and 5-HT in other experiments (Whitman et al., 1992). Thus, it is possible that there is a sex difference in the ability of 5-HT to inhibit flank marking.

There is considerable evidence that 5-HT can inhibit aggression in a variety of species (Eichelman, 1990) and there is recent evidence in male hamsters that 5-HT may inhibit aggression facilitated by VP. Systemic injection of fluoxetine inhibits offensive aggression stimulated by injection of VP in either the AH or VLH (Ferris and Delville, 1994). No studies have been conducted in females to indicate whether VP and 5-HT interact to regulate aggression. However, recent studies in

hamsters suggest that there is an important sex difference in the role of the 5-HT system in regulating aggression (Joppa et al., 1997). In males, intracerebroventricular injection of a 5-HT agonist significantly reduced inter-male offensive aggression, but did not reduce offensive aggression in females. It will be interesting to determine whether the inability of 5-HT agonists to inhibit aggression in females is the result of the inability of 5-HT to inhibit the facilitatory effects of VP on aggression in the AH.

VP and sex differences

There are dramatic and unusual sex differences in the expression of flank marking and aggression in hamsters (Albers and Prishkolnik, 1992; Payne and Swanson, 1970;). Unlike most species, female hamsters scent mark at higher levels than males and exhibit higher levels of aggressive behavior. It seems somewhat unlikely that the sex differences in these behaviors are the result of sex differences in the vasopressinergic system. Examination of VP-IR in a variety of limbic regions in male and female hamsters has not revealed the sex differences that have been reported in other rodent species (Delville et al., 1994; Hennessey et al., 1994). The only evidence for a sex difference in VP-IR in hamsters comes from one study (Delville et al., 1994) which reported a sex difference in VP-IR. Males had 50% more VP-IR in the supraoptic nucleus than females. However, another study (Hennessey et al., 1994) found no sex differences in VP-IR in the supraoptic nucleus. There is also no evidence that sex differences in the amount of flank marking result from sex differences in the response to VP since the amount of flank marking stimulated by injection of several different concentrations of VP into the MPOA-AH was similar in male and female hamsters (Hennessey et al., 1994). While the possibility that sex differences in flank marking and aggression result from sex differences in the vasopressinergic system cannot be excluded, the existing data do not provide strong support for this hypothesis. At present it seems more likely that the sex differences observed in these behaviors are the result of sex differences in the effects of NE and/or 5-HT on the release of, or response to, VP. There is evidence that sex differences exist in the functioning of NE and 5-HT systems in the MPOA-AH of a number of rodent species (De Vries, 1990), however it will be important to examine whether sex differences in these systems occur in hamsters.

OT and sexual receptivity

There is considerable evidence that OT plays a significant role in the regulation of social behavior in several species. For example, in female prairie voles central administration of OT inhibits sexual receptivity but enhances social behaviors associated with sexual receptivity (Witt et al., 1990). In rats, central administration of OT increases, and an OT antagonist reduces, sexual receptivity (Arletti and Bertolini, 1985; Caldwell et al., 1986; Gorzalka and Lester, 1987; Witt and Insel, 1991).

Studies on the role of OT in the control of female reproductive behavior in the hamster have focused on the MPOA-AH and the ventromedial hypothalamus (VMH). In hamsters, both the MPOA-AH and VMH contain OT-IR fibers (Whitman and Albers, 1997). Microinjections of OT into either the MPOA-AH or VMH of ovariectomized, estradiol-treated hamsters stimulate high levels of sexual receptivity (Whitman and Albers, 1995) (Fig. 6). The OT-responsive region appears to extend from the posterior medial preoptic area through the anterior hypothalamus. This region overlaps at least a portion of the area where VP stimulates flank marking. The OT-responsive region in the VMH appears to be localized in the posterior aspect of the nucleus. The OT-responsive regions in the MPOA-AH and VMH are anatomically separable since injection of OT between the AH and VMH is not effective in stimulating sexual receptivity. In both the MPOA-AH and VMH, OT significantly increases the duration of lordosis when compared to saline injected controls. OT injected into the MPOA-AH or VMH increases sexual receptivity in a dose-dependent manner when the hamsters are tested immediately following injection, as well as 30 and 60 min following injection. Further support for the hypothesis that OT plays a critical role in regulating sexual receptivity was provided

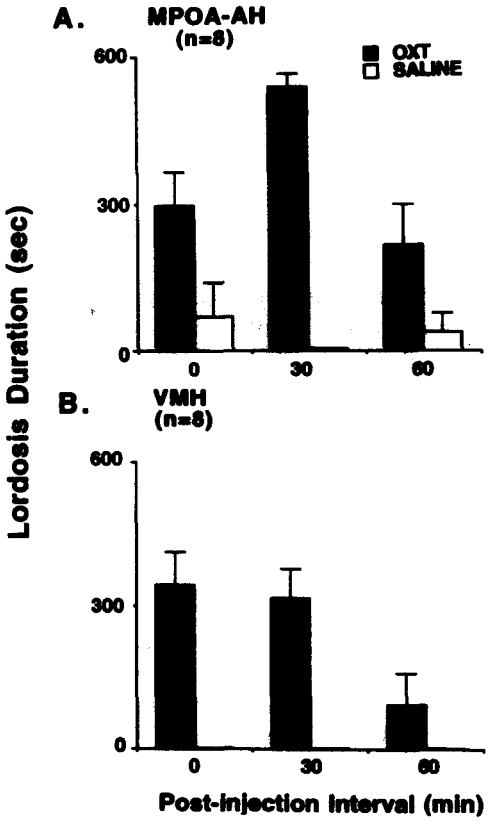

Fig. 6. The duration of lordosis after injection of OT and saline into the MPOA-AH and the VMH of ovariectomized hamsters given estradiol benzoate. Behavioral tests were conducted 0, 30 and 60 min after peptide injection. The duration of lordosis was significantly higher in hamsters receiving OT than in those receiving saline in both sites. From Whitman and Albers, 1995; used with permission from *Brain Research.*

by studies employing an OT antagonist. Injection of the OT antagonist into the MPOA-AH or VMH of ovariectomized hamsters given both estradiol and progesterone significantly inhibited sexual receptivity. The inhibition of sexual receptivity was most pronounced in the MPOA-AH where the OT antagonist reduced the duration of lordosis behavior by approximately 80%. Taken together these data indicate that OT plays a critical role in the regulation of sexual receptivity in hamsters by its action within the MPOA-AH and the VMH.

OT and ultrasonic communication

Ultrasonic communication is a means of social communication that appears to aid in the initiation and structuring of mating. The MPOA-AH is an important region in the control of ultrasonic communication, and OT injected into the MPOA-AH influences the expression of this behavior in female hamsters (Floody et al., 1995). Injection of OT, but not VP, into the MPOA-AH significantly increases the rate of ultrasound production. It will be important to determine whether injection of an OT antagonist into the MPOA-AH can block or reduce ultrasonic communication. If so, OT within the MPOA-AH may be an important component in the neurochemical regulation of this behavior.

OT and flank marking

Recent data indicate that social context or the social history of an animal may alter the behavioral response to OT. OT stimulates little or no flank marking in hamsters tested in solitary conditions (Ferris et al., 1984; Albers and Ferris, 1986). However, in at least some social conditions, OT is capable of stimulating flank marking (Harmon et al., 1996). In female hamsters that have established dominant/subordinate relationships, injection of OT into the MPOA-AH of the dominant female stimulates high levels of flank marking when she is tested during a social encounter with a subordinate, but OT stimulates little or no flank marking when she is tested alone. The ability of OT to stimulate flank marking is not simply the result of testing the hamster with another individual. When OT is injected into the MPOA-AH of a female tested with another female with whom she has not previously interacted, little or no flank marking is observed. These data suggest the possibility that social history or context can alter the behavioral response of specific brain sites to OT.

Conclusions

VP and OT appear to play a major role in the regulation of a variety of social behaviors in Syrian hamsters. Both VP and OT can act at more than one site to influence the same behavior and at the same

site to influence different behaviors. The ability of social factors and hormones to influence several behaviors also appear to result from their effects on neurons that release or respond to VP and OT. The existing data suggest that social and hormonal factors influence VP and OT activity in two basic ways. First, social factors and hormones appear to alter the type or frequency of behavior elicited in response to these peptides. For example, gonadal hormones seem to modulate the expression of flank marking by modulating the levels of flank marking stimulated by VP in the MPOA-AH. One way gonadal hormones may modulate the response to VP is through altering VP binding in this region. There is also evidence that social context or social history can influence the response of the MPOA-AH to these peptides. In female hamsters hormonally prepared to mate, the behavioral response to VP in the MPOA-AH is blocked when the female is tested with a male hamster but not when she is tested alone. Another example comes from studies on OT. OT injected into the MPOA-AH produces little or no flank marking in female hamsters tested alone or with an unfamiliar female. However, OT produces high levels of marking in dominant hamsters paired with their subordinate partner. It will be interesting to determine the extent to which social and hormonal events influence social behavior by modifying the response to VP or OT within specific elements of the neuronal circuits controlling these behaviors.

Second, social factors and hormones could alter social behaviors controlled by VP and OT by altering the release of these peptides. While examination of this hypothesis has been limited by the difficulty in measuring changes in peptide release in CNS neuronal circuits, there is some indirect evidence to support this possibility. After stable dominant/subordinate relationships have been established, subordinate hamsters have lower levels of VP-IR in the AH than do their dominant counterparts or controls that have not established dominant/subordinate relationships. These data suggest several interesting possibilities about how social experience might influence the expression of social behavior by modifying the release of VP and OT. Social stimuli might trigger rapid changes in the release of peptides within elements of the neuronal circuits (e.g. MPOA-AH) that control one or more social behaviors. For example, the introduction of an intruder might stimulate or inhibit the release of VP or OT and thereby change the behavioral response of the resident hamster. The amount of peptide released in response to the intruder might determine the magnitude or type of social behavior elicited. For example, aggression might be stimulated in response to the release of low concentrations of VP into the MPOA-AH. The release of higher concentrations of VP might stimulate the resident to flank mark in response to the intruder. Another interesting possibility is that the social history of an individual serves to determine the amount of peptide available to be released in response to a social stimulus such as an intruder. The amount of peptide available for release within the MPOA-AH may be determined by whether the resident has previously had social interactions with that individual and the nature of those interactions. For example, different concentrations of VP would be available for release in response to the intruder depending on whether the resident had previously established a dominant or subordinate relationship with that individual. Such a mechanism may provide for 'social memory' and help explain how the social history of an individual influences its response to the social stimuli it encounters in the future.

In summary, social behaviors controlled by VP or OT may be influenced by how social factors and hormones alter the release of, or the response to, these peptides within key elements of the neural circuitry controlling specific social behaviors. A number of factors could be responsible for modulating the release of, or response to, VP or OT including the action of other neurochemical signals such as NE or 5-HT. It will be interesting to determine the extent to which these mechanisms can account for the rapid and dramatic changes that are observed in social behavior.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
Progress in Brain Research, Vol. 119

CHAPTER 5.2

Vasopressin, gonadal steroids and social recognition

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Rodents exposed for a short amount of time to conspecific juveniles spend less time investigating familiar than unfamiliar juveniles. This is based on the formation of an olfactory image of juveniles, which involves an androgen-dependent vasopressinergic pathway in males, as demonstrated by the ability of the vasopressin receptor antagonist dPTyr(Me)VP to block social recognition in intact male but not in female and castrated rats and mice. The involvement of sexually dimorphic vasopressinergic neurons appears to be dependent on the processing of social olfactory cues by the vomeronasal organ since removal of this organ in male rats mimics the effects of castration. These findings are discussed in relation to the role of vasopressin in learning and memory.

Introduction

The neuropeptide arginine vasopressin (VP) has been implicated in learning and memory. The first demonstration of this activity was obtained by de Wied and colleagues in the mid-1960s. They showed that the faster extinction of a conditioned avoidance response, which was observed in rats after ablation of the neurohypophysis, was reversed by administration of an extract of the neurointermediate part of the pituitary (De Wied, 1971). The active component of this extract was subsequently identified as vasopressin. Various fragments of vasopressin, including the desglycinamide DGVP which is devoid of hormonal effects, were found to induce behavioural changes similar to those induced by VP. A physiological role for endogenous vasopressin in learning and memory was further suggested by a series of experiments on rats with a genetically deficient vasopressin system (Brattleboro rats) and rats made temporarily deficient in vasopressin by central administration of an antiserum to VP. In both instances, deficiency of VP was associated with an impaired performance in a learning task, an effect which was opposite to the memory enhancing action of VP (van Wimersma Greidanus et al., 1975, 1986).

This impressive series of results on the memory enhancing effects of VP was challenged in the early 1980s by the demonstration that behaviourally-active doses of VP still retained potent vasopressive activity and that when the vasopressor activity of VP was blocked by a specific antagonist of the vasopressor receptor of VP, dPTyr(Me)VP, this blockade was associated with an abrogation of the behavioural effects of VP (Le Moal et al., 1981). These findings were interpreted to suggest that the memory enhancing effect of VP was secondary to the visceral arousal produced by systemic administration of this peptide, or to the activation of the brain substrate of this visceral arousal when VP was administered centrally.

The question of finding out whether vasopressin has direct effects on learning and memory or these effects are secondary to changes in arousal fuelled a vivid scientific controversy between the Utrecht group and the Bordeaux group, which lasted for nearly one decade. This controversy turned out to be quite productive since it was a strong impetus for the quest of new leads for elucidating the mechanisms of the behavioural effects of VP. At the beha-

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vioural level, the controversy was not easy to resolve since the acquisition and extinction of active and passive avoidance responding, which were commonly used for assaying the memory enhancing effects of vasopressin, were inevitably contaminated by performance factors such as arousal and emotionality. In an attempt to circumscribe these problems, we decided in the late 1980s to look for a more natural test of memory in rodents and we eventually selected for this purpose a test of social memory. The objective of this review paper is to show how the use of this test enabled us to get some insight of the role of androgen-dependent vasopressinergic neurons in the processing of olfactory cues sensed by the vomeronasal organ in rodents.

Characteristics of social memory

The test of social memory was originally proposed by Thor and Holloway (1982). It is based on the tendency of rodents to investigate more intensely unfamiliar than familiar conspecifics. When a rat is presented with a conspecific, it engages in bouts of intense investigation of the stimulus animal. This investigation consists mainly of sniffing, nosing, following and grooming. The use of juvenile conspecifics as social stimuli limits the likelihood of intrusion of aggression and sexual behaviour in the repertoire of the test animal. Presentation of the juvenile for a limited amount of time (5 min usually) serves a similar objective.

When an unfamiliar juvenile is introduced for the first time into the home cage of an adult rat, it is intensely investigated. If it is presented for a second time shortly after the first encounter, it receives much less attention. This is not due to satiation since presentation of a novel juvenile at that time triggers the full sequence of investigation. If the already familiar juvenile is presented after an interval of 1–2 h, it is investigated as if it is no longer recognized.

Based on these findings, it is possible to use variations in the duration of social investigation as an index of social memory of the juvenile. The fact that an adult animal spends less time investigating a previously presented juvenile is interpreted to indicate that the adult animal has formed a memory of the juvenile and that this memory is still present. Conversely, the fact that an adult animal spends as long investigating a previously presented juvenile as on the first presentation is interpreted to indicate that the memory the adult has formed of the juvenile is gone. This form of memory is short-lasting since it is present in adult rats for only about 40 min. It can be prolonged by repeated exposure to the same juvenile and it is impaired by presentation of a different juvenile in the interval between two presentation of the original juvenile (retroactive interference) (Thor and Holloway, 1982; Dantzer et al., 1987).

The sensory stimuli on which the formation of social memory is based are represented by olfactory cues, as demonstrated by the possibility of replacing presentation of the juvenile by urine or soiled bedding from the cage in which the juvenile has been housed (Sawyer et al., 1984). The exact cognitive dimension which is involved in the test of social memory is not easy to identify since the procedure does not allow to tell the difference between social recognition which is simply based on familiarity and individual recognition which is based on discrimination of intrinsic characteristics of the individual under investigation. It is likely that most of the behavioural changes that occur in rodents exposed to different conspecifics are based on the dimension of familiarity. This feature is important since, as we will see later, the chemoceptive detection of familiar versus unfamiliar social cues is mediated by the accessory olfactory tract whereas the recognition of individuality depends on the main olfactory neural pathways.

Effects of vasopressin on social memory

In accordance with what was predicted based on its memory enhancing effects, administration of VP immediately after the first presentation of a juvenile enhanced the duration during which the memory of this particular juvenile was held. More specifically, rats treated with VP spent less time investigating the same juvenile when it was presented 2 h after the first presentation, at a time at which there is normally no recognition (Dantzer et al., 1987). This effect was not due to toxic effects of the peptide treatment since there was no decrease in social investigation when a different rather than a

previously explored juvenile was presented to VP-treated rats. It was not due either to an acquired aversion to the olfactory cues of the juvenile since administration of angiotensin II, at a dose which had the same aversive properties as VP, had no effect on social memory.

The facilitatory effect of VP on social memory was mediated by the vasopressor receptors of VP since it was blocked by pretreatment with the vasopressin receptor antagonist, dPTyr(Me)VP. VP had the same effects on social memory when it was injected into the lateral ventricle of the brain and the lateral septal brain area instead of subcutaneously, indicating that the facilitation of social memory is mediated by central vasopressin receptors (Dantzer et al., 1988). In the same manner, microdialysis administration of vasopressin into the mediolateral septum improved social recognition in homozygous Brattleboro and normal Long–Evans rats (Engelmann and Landgraf, 1994). Analogs of VP devoid of hormonal activity (DGVP and VP 4–8) were as effective to facilitate social memory as VP itself (Popik et al., 1991).

Oxytocin (OT), which acts in an opposite way to vasopressin in other memory tasks, was found to impair social recognition (Dantzer et al., 1987). Intracereboventricular injections of OT, in the dose range of 1 fg to 1 ng per rat, had the same effect, although doses larger than 1 ng/rat were ineffective (Benelli et al., 1995). The possibility of a differential activity of OT according to the dose was explored by Popik et al. (1991, 1992). When administered subcutaneously, very low doses of OT shared the same activity as VP whereas higher doses had the opposite effect (Popik et al., 1992). Comparison of the effects of OT with those of OT-related peptides revealed that the impairing effects of OT on social recognition resided in the 5–7 region whereas its facilitating effects resided in the 8–9 region of the OT molecule (Popik et al., 1996).

Role of endogenous vasopressin in social recognition

Administration of the vasopressor receptor antagonist dPTyr(Me)VP was found to have effects opposite to those of VP, in the sense that rats treated with this peptide behaved in response to a familiar juvenile as if this juvenile was no longer recognized, i.e. they spent as long investigating the juvenile as on the first presentation. This effect was observed whether the antagonist was administered systemically or into the lateral septum of the brain (Dantzer et al., 1987, 1988; Engelmann and Landgraf, 1994). Since the antagonist is able to cross the blood–brain barrier, these effects were interpreted to suggest that brain vasopressin is involved in social recognition.

Blocking endogenous vasopressin by intracerebroventricular administration of an anti-vasopressin serum had the same effect as the V_1 receptor antagonist, i.e. it impaired social recognition (van Wimersma Greidanus and Maigret, 1996). The same result was obtained when the antiserum was injected into the dorsal or ventral hippocampus, and the dorsal septal area.

Role of gonadal steroids

A demonstration of a role of endogenous VP is important since it provides a case for investigating which brain vasopressinergic pathway is involved in the phenomenon under study. The distribution of VP in the brain of rodents has the peculiarity of including androgen-dependent and androgen-independent pathways, as demonstrated by De Vries and colleagues in the early 1980s. Using immunocytochemistry, they found that the density of VP containing fibres which originate from the bed nucleus of the stria terminalis (BNST) and the medial amygdala (MA), and project to the lateral septum (LS) is higher in males than in female rats, from the 12th postnatal day (De Vries et al., 1984). This is due to the presence of testosterone, which exerts both organizational and activational effects on this pathway. The number of VP neurons in the BNST and the density of their projections to the LS can be increased in female rats by neonatal treatment with testosterone. Conversely, they are reduced in male rats subjected to castration during the neonatal period. In addition, VP immunoreactivity is dramatically attenuated following castration at adulthood in males, whereas steroid replacement restores it to normal.

The sex difference in VP staining that is observed

in the rat brain reflects more VP synthesis in the BNST and MA of males, since in situ hybridization techniques show a higher number of cells that are labelled for propressophysin mRNA and a higher number of grains per labelled cell in males than in females. However, the decrease in VP mRNA develops more rapidly after castration than the corresponding decrease in VP immunoreactivity, suggesting that immunoreactive VP is not readily mobilized (Miller et al., 1989).

Based on this sexual dimorphism in the distribution of VP-containing neurons in the brain of rats, we predicted that if androgen-dependent vasopressin is involved in social recognition, females and castrated male rats which are deficient in VP projecting to the LS should form a social recognition that is independent of VP and therefore insensitive to the blocking effect of the VP antagonist. In accordance with this prediction, administration of dPTyr(Me)VP to females and castrated male rats no longer opposed the reduction in social investigation that is normally observed in animals exposed for the second time to a previously explored juvenile (Bluthé et al., 1990). Furthermore, testosterone replacement in castrated male rats fully restored sensitivity of social recognition to blockade of vasopressinergic neurotransmission. The ability of female rats to recognize previously presented juveniles appears to be dependent on endogenous OT since it was blocked by the intracerebroventricular administration of the OT receptor antagonist des-Gly-NH_2 $d(CH_2)_5(Tyr(Me)^2Thr^4)OT$ (Engelmann et al., 1998). However, the sexual specificity of the involvement of brain OT in social recognition is doubtful since administration of anti-OT serum into the ventral hippocampus of male rats also impaired social recognition (van Wimersma Greidanus and Maigret, 1996).

The influence of gonadal steroids on the involvement of VP in social recognition is not restricted to rats. Intact male DBA2 mice form a social memory of juvenile conspecifics which lasts for at least 1 h and can be prolonged by VP or impaired by dPTyr(Me)VP (Bluthé et al., 1993). Castrated male mice are still able to recognize juvenile conspecifics but this recognition is no longer sensitive to dPTyr(Me)VP.

The characteristics of social recognition are different in male and females. Females spend much less time investigating juveniles than males, but the social memory they form of juveniles lasts longer (Bluthé and Dantzer, 1990). Castrated male rats behave like females and testosterone replacement restores a male pattern of social exploration and recognition (Bluthé et al., 1990). Chronic intracerebral infusion of vasopressin makes castrated rats behave like intact male rats, whereas chronic intracerebral infusion of the vasopressin antagonist dPTyr(Me)VP makes intact male rats behave like castrates (Bluthé and Dantzer, 1992).

Role of the vomeronasal system

In addition to their insensitivity to the effect of the vasopressin receptor antagonist on social recognition, castrated male rats were found to display a temporary disruption of social recognition when they were tested 1 week after castration, but not when they were tested every other day following castration (Bluthé et al., 1990). These results are strongly suggestive of a gradual shift from a vasopressinergic to a non-vasopressinergic mediated neurotransmission in the neural circuit involved in social recognition, following the drop in plasma testosterone levels due to castration.

An important sensory organ for social communication in rodents is the vomeronasal organ. This organ is involved in the processing of non volatile chemoceptive stimuli such as sex pheromones. As mentioned earlier, social investigation typically involves close following and licking of the anogenital area of the social stimulus, and this is done by the adult rat with its head bowed to the side. This posture and the lack of sniffing at distance are strongly suggestive of the use of the vomeronasal organ in sensing non volatile olfactory cues from the juvenile. The vomeronasal organ projects to the accessory olfactory bulbs and from there to the medial and the posteromedial nucleus of the amygdala, with a relatively minor input to the BNST. The medial nucleus of the amygdala in turn sends massive projections to the BNST and the medial preoptic nucleus of the hypothalamus. The vomeronasal pathway is sexually dimorphic in

rats. The vomeronasal organ and its projection areas are larger in adult male rats than in females, and this difference is dependent on circulating androgens.

Based on the influence of gonadal steroids on the role of vasopressin in social recognition and the sexually dimorphic features of the vomeronasal system, we proposed that the involvement of vasopressin in social recognition is critically dependent on the processing of olfactory cues of juveniles by the vomeronasal organ. To test this hypothesis, male rats were submitted to ablation of the vomeronasal organ and their ability to recognize previously presented juveniles was tested with and without blockade of vasopressinergic neurotransmission by dPTyr(Me)VP (Bluthé and Dantzer, 1993). Ablation of the vomeronasal organ decreased the duration of social exploration and temporarily impaired social recognition. This was not due to physical debilitation since vomerectomized rats were perfectly normal in their investigatory behaviour towards juveniles. After restoration, social recognition was no longer sensitive to the disrupting effects of the vasopressin receptor antagonist, despite unchanged levels of circulating androgens. These results can be interpreted to suggest that the vomeronasal pathway represents the main sensory system by which male rats process and store socially relevant cues.

Conclusion

The involvement of vasopressin in social recognition appears to be specific of social cues since object recognition is not impaired by infusion of dPTyr(Et)VP into the lateral septum, in contrast to social recognition (Everts and Koolhaas, 1997). The sexual dimorphism observed in the disrupting effects of the vasopressin receptor agonist dPTyr-(Me)VP on social recognition does not apply to the effects of vasopressin on social recognition. Administration of vasopressin itself prolongs social recognition in intact males as well as in females and castrated male rats. These results point to the existence of two functionally different vasopressinergic systems in the brain of rodents, an androgen-dependent vasopressinergic system which is involved in the processing of socially relevant olfactory cues, and an androgen-independent system which is involved in modulation of arousal.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
Progress in Brain Research, Vol. 119

CHAPTER 5.3

Neurohypophyseal peptides and social recognition in rats

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An encounter between rats results in bouts of social investigation consisting mainly of sniffing, nosing, following and grooming. The assessment of social recognition is based on the tendency of rodents to investigate unfamiliar conspecifics more intensely, than familiar ones. In the laboratory an immature conspecific is normally used as the social stimulus because the use of juveniles eliminates possible sexual and/or aggressive behaviors of the rat whose memory is assessed. When a juvenile is presented for the first time, it is intensely investigated. A second presentation shortly after the first one elicits less attention. This is not due to satiation or fatigue, since the presentation of a novel juvenile triggers the full sequence of investigation. Social recognition is defined as a specific decrease in social investigation during the second encounter of the same individual. This form of memory is short lasting (<40 min) and based on the olfactory characteristics of the stimulus animal. Social memory is prolonged by repeated exposure to the stimulus juvenile rat and is impaired by retroactively interfering stimuli. It can be facilitated by vasopressin and derivatives as well as by several other memory facilitating compounds, and, depending on the dose, attenuated or facilitated by oxytocin and derivatives. Ethologically oriented memory tests, that are based on olfactory characteristics of the information to-be-remembered, have an advantage over 'classical' ones: they estimate behavioral patterns which are important to an animal and not only to the investigator. Social memory paradigms can reveal information about memory processes in animals that is relevant for memory deficits in humans.

Social behavior

The social way of life is a quantum jump in the biological organization of life. There is a strict limit to what a single animal or human is able to accomplish. In contrast, a society can evolve to much greater complexity by the processes of differentiation and integration, even without any increase in the powers of its individual members. Living in a society usually enhances the probability of survival of the subjects in a number of ways, for instance by securing protection against predators or by facilitating mating and reproduction (Wynne-Edwards, 1972). Rats form family groups consisting of a female and her young; such groups are bound together during the period of dependence of the young on their mother. But rats are also in other ways social animals in that they gather in groups whenever they have the opportunity. Wild rats, for example, live in colonies within which nesting sites and feeding grounds are shared (Calhoun, 1962).

A feature of the families and societies is social homeostasis. For example, building nest sites for the young and adjusting the number of individuals accordingly to environmental resources, both enhance the probability of survival of the closest relatives. One of the basic conditions for the establishment of a social structure among gregariously living vertebrates, is their innate tendency to engage in social relations (Kalkowski, 1967). The social homeostasis is controlled by social activities or behaviors. The term 'social behavior' is used here to describe all activities which influence, or are influenced by other members of the same species, including behavior that tends to bring individuals together as well as conflict behaviors.

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Rats display a set of social behaviors that consist of about 60 precisely described acts and body postures (Grant, 1963; Grant and Mackintosh, 1963; Silverman, 1965). The common feature of social activities is their dependence on the presence of another animal of the same species. The encounter elicits social behaviors, but can also lead to a number of non-social activities. The social behaviors of laboratory rats can be divided into four main categories:

- *Social explorative behavior* consists of all activities in which the rat investigates the other by smelling and licking or by using its whiskers. This category includes exploration of the partner and, particularly anogenital investigation (Fig. 1). Since the rat's urine may convey the chemical messages concerning subject's individuality (Singh et al., 1988), anogenital investigation may play a crucial role providing information about the conspecific.
- Rats as compared to other species, spend much time in *social contact*, which consist of activities such as crawling under, walking over, nosing, mounting and social grooming.
- *Attentive behaviors* include such activities as approaching and following the conspecific, or generally all behaviors characterized by walking in the direction of another rat.
- *Aggressive-like behaviors* hardly occur in an established group of rats. The behaviors include biting, kicking and fighting.

It is worth to note that most of the social behaviors are displayed in clusters or sequences, resulting in a specific structure of social behavior.

As a society becomes more efficient and spatially structured, its members become more specialized into roles or castes and their relationships become more precisely defined by means of better communication. To achieve a permanent and stable social structure, the rats' society, as other societies, is organized in such a manner that its members differ from each other and have distinct roles, which are related to age, sex, sexual receptivity stage, position in social hierarchy and so on. It is obvious that some roles are confined to a restricted number of members, since any attempt of others to take a role that is occupied or could not be shared may

Fig. 1. Anogenital investigation behavior. The rat on the left investigates anogenital areas of the subject on the right. Several data indicate that through this behavior a rat acquires olfactory information about the conspecific.

cause social conflicts. The society is protected against occurrence of such conflicts, by, among others, mechanisms allowing easy recognition of the member(s) individuality and by mechanisms allowing appropriate behavior towards the conspecific. Hence, the members of a society need special sets or systems of behaviors to express and to recognize relations among them. Within rodent societies olfactory messages play a crucial role. To recognize the members of another clan, rats excrete a set of odors, which reflect their own olfactory characteristics (Krames, 1970; Carr et al., 1976), also called 'odor signatures' (Wilson, 1978). But the olfactory channel of communication may transmit in addition messages concerning age (Thor, 1979), sex (Brown, 1977; Johnston and Rasmussen, 1984) (including the sexual condition (Stern, 1970; Gilman and Westbrook, 1978; Drewett and Spiteri, 1979)), group membership (Krames and Shaw, 1973) and position in the social hierarchy (Krames and Carr, 1969). Rats are also able to excrete odors reflecting their emotional state, e.g. so called 'alarm pheromones', produced during stress (Sawyer, 1980), and 'frustration odors', appearing in situations where reward is expected, but not offered (Morrison and Ludvigson, 1970). Interestingly, other rats may be conditioned to both of these odors. All of these odors may be perceived and then successfully used to allow an animal to behave properly during encounters. However, correct usage requires a memory of the particular olfactory message, which is among others the basis of social recognition.

Social memory

The term 'social memory' is defined here as the storage and retrieval of information about the individuality and the present condition of the conspecific. Social memory can deal with all kinds of information about a subject. It can be an information of a general nature (like: that subject is an alpha male) or concerning the temporary conditions (like: this female has been sexually receptive). In this respect the knowledge about the conspecific, rather then skills gained as an outcome of the encounter (imitation learning (Miller and Dollard, 1943)), is of importance. The physical presence of the subject which is to remembered is unnecessary, as his or her olfactory cue, for example, may serve as a sufficient stimulus.

Social memory, like other kinds of memories, requires learning preceding the formation of the internal representation of the conspecific (Wixted, 1989). Since rats use mostly the olfactory channel of communication, (one of the earliest appearing in their life (Small, 1899)), learning in this respect depends on acquisition of olfactory information. Accordingly, experimental manipulations producing an olfactory deficit decrease this type of learning (Alberts, 1974). There are several examples of social olfactory learning, including kin recognition, which under laboratory conditions has been studied first in spiny mice, *Acomys cahirinus*, (Porter et al., 1978; Porter and Wyrick, 1979). It may be deduced that the phenomenon of kin recognition is of great importance both to animals and humans. From a sociobiological point of view, the well-being of the close family of the individual will be particularly considered. From a genetic perspective, the species would benefit if its pool of genes survives, which is accomplished, among other factors, by mating with non-kin rather than with close relatives. Rats have a native ability to differentiate close kin from other conspecifics (Hepper, 1983; Halpin, 1986; Bolhuis et al., 1988). This differentiation is based mainly on olfactory cues. It has been suggested that this ability is acquired in utero as well as post partum (Hepper, 1983).

Unique olfactory characteristics are essential for some complex social systems, such as territory. It might be assumed that each member of the rat society is familiar with the odor characteristics of each other. Within a colony, rats behave amicably towards other rats and little fighting occurs (Von Steiniger, 1950). However, intruders are usually attacked by the colony members (Calhoun, 1948; Von Steiniger, 1950). Visiting strange rats compete for territory. The necessity of an accurate reaction needs rapid recognition and that may explain why every encounter between rats starts with intensive anogenital investigation and other kinds of recognition sniffing (Barnett, 1964). A strange male is usually attacked by the dominant so-called alpha male (Alberts and Galef, 1973; Luciano and Lore,

1975; Flannelly and Thor, 1978). The consequences of 'strangeness' might however be avoided, when the intruder (being, as a juvenile, immune to attack) had an encounter with the dominant male in the past (Thor and Carr, 1979). In addition, the odor of a strange male has specific effects on female's behavior. It may block pregnancy (Bruce, 1959; Bruce and Parrott, 1960; Parkes and Bruce, 1961), and this 'Bruce effect' depends also on the unfamiliarity with the olfactory cues produced by the stranger (Halpin, 1986; Brennan et al., 1990). In those examples, the odor of the intruder is new, that is, unknown for the particularly behaving rats, and its further or previous experience may change the behavior of the recipients.

Not only the inborn olfactory characteristics are important for rats. An example here may be an acquired odor, which may enhance the conspecific's probability of survival. The phenomenon called social transmission of food preferences (Galef and Wigmore, 1983) is based on anecdotal observations taken from rats living in semi-natural conditions, and concerns socially mediated poison avoidance (Von Steiniger, 1950). Rats display food neophobia, and will ingest a new food with a high attention. However, if a healthy conspecific bearing the odor of new food is presented to an 'observer' rat, such a presentation will facilitate his (or her) future consumption of this particular food. On the other hand, if the 'demonstrator' displays signs of illness or death, almost permanent avoidance of the food associated with that odor occurs. This kind of learning is rapid and the memory is strong, which may be related to its biological significance. Thus, wild rats need only a few minutes of sniffing the mouth and/or body of the 'demonstrator' to remember that odor for a long time.

Social memory paradigms

There are several approaches to study the olfactory, individual recognition (Halpin, 1986). They are listed and briefly described here.

(a) *Habituation-discrimination* depends on differential responses to a familiar odor as compared to a novel odor (Halpin, 1974). Generally, the procedure follows the presentation to the test animal of odor from one individual (A) until habituation occurs; the test animal is then confronted simultaneously with the same odor (A) and with the odor from a second individual (odor B). The first part of the procedure is considered as the habituation phase while the second part is the discrimination phase. If odors A and B are different, and if the test animal has habituated to odor A, then during the discrimination phase odor B should elicit a stronger response than odor A. It has been shown for instance, that exposure to an odor may enhance subsequent neural responses of the rat pups olfactory bulbs to that odor, as revealed by enhanced ^{14}C-labelled 2-deoxy-D-glucose uptake (Coopersmith and Leon, 1984).

(b) *Positive reinforcement* (Bowers and Alexander, 1967), is based on the fact that animals can be trained to respond preferentially to one odor as compared to another. Typically two odors from different individuals are presented to the test animal and the animal is rewarded for responding to one of the odors but not for that to the other. If, after a number of trials, the animal learns to discriminate between the two odors, it is assumed that the two odors are different and that the animal could distinguish them. The advantage of this technique is that, unlike the habituation-discrimination method, it can provide information on whether animals can distinguish between odors of equal familiarity. A disadvantage is that in this paradigm the animals are tested under rather artificial conditions which may have little relevance to the natural habits or behaviors of the animals.

(c) *Differential responses to conspecific odors.* The procedure usually involves the exposure of a test animal to odors from two or more conspecifics that do not differ in either age or sex. If the animal responds differently to the different odors, it is assumed that this is because the odors are different (Carr et al., 1970). In most cases, however, the ability to distinguish between odors is based, at least in part, on the degree of familiarity with the odors. Typically, the test animal is exposed to an odor from a familiar conspecific and its responses to this odor are compared to those elicited by the odor of an unfamiliar conspecific. The responses depend on the previous exposure to one odor as compared to the novelty of a second odor (Porter and Moore, 1981). The social recognition paradigm, used in the

experiments described later, may serve as an example of differential responses to conspecific odors.

(d) *Discrimination of own odor versus conspecific odor.* This technique is similar to the one discussed before, and probably depends on the ability to discriminate between odors of unequal familiarity. Asymmetry in familiarity serves as the basis for discrimination (Brown, 1973; Porter et al., 1981). For example, when tested at 12 days of age, rat pups preferred a bedding odor from their nest; 20-day-old pups preferred a strange bedding (Carr et al., 1979).

(e) *Primer effects.* Those studies, particularly the pregnancy block or 'Bruce effect', are not generally used to demonstrate the existence of individual odors, but they nevertheless provide circumstantial evidence for such odors. In species that show the Bruce effect, a female that has been recently inseminated can have her pregnancy blocked by exposure to the odor of a strange male that is genetically dissimilar to the original mate. Unequal familiarity with the two odors appears to play a role in the discrimination (Parkes and Bruce, 1961). It has been found that the Bruce effect is contingent upon noradrenalin activation and mating, and lasts for several weeks (Brennan et al., 1990). The suggested mechanism involves Hebbian (association-dependent) changes in synaptic efficacy at dendritic synapses in the accessory olfactory bulb.

The *social recognition test*, used in experiments described here, is based on the observation that under laboratory conditions an adult, i.e. sexually mature male rat, will investigate a conspecific juvenile less on repeated encounter, provided that the time interval between encounters is short, i.e. no longer than 30 min (Thor and Holloway, 1982). This phenomenon is however absent, if another juvenile rat is presented on the second encounter. That specifically diminished interest is considered as the social recognition of the conspecific (Fig. 2). Recently, a social discrimination paradigm was developed in which, during the second encounter the initial and the novel juvenile were presented simultaneously (Engelmann et al., 1995). Several findings indicate that social recognition is based on olfactory cues emitted by the juvenile (Sawyer et al., 1984; Popik et al., 1991a). It has been shown that the urine of rats may contain bio-degradation products of the class I transplantation antigens (glycoproteins) on which the unique characteristics of the subject may depend (Singh et al., 1988). Since those antigens display an extreme species polymorphism (Singh et al., 1987), they are considered as molecules of 'olfactory signatures', mentioned above. Highly inbred strains of rats are thought to possess the same genetic make-up, and this may explain why different inbred rats are not discriminated (Brown et al., 1987). Thus, the urine

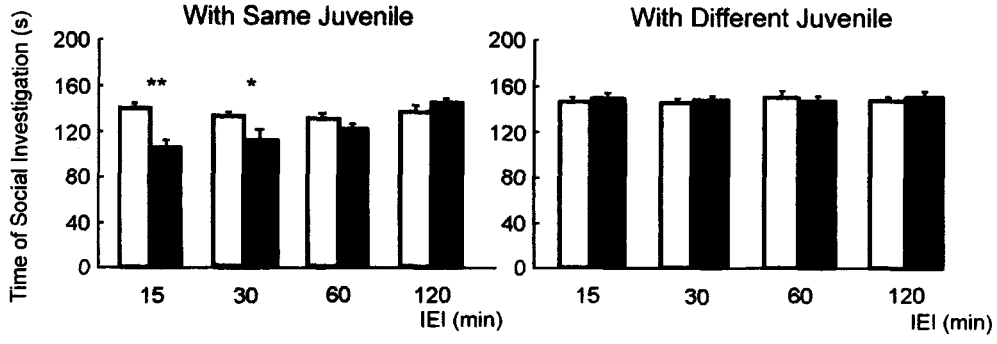

Fig. 2. Time dependence of social recognition. Presented are mean and SEM times of social investigation of adult rats during two separate encounters with the same or a different juvenile. The first encounters are represented by open bars and the second encounters by black bars. Both encounters were separated by various inter exposure intervals (IEI). It is evident that the memory for the conspecific is short lasting, and that there is no decrease of social interest when a different juvenile is presented on the second encounter. Asterisks indicate a difference from first encounter ($*P < 0.05$, $**P < 0.001$). (From Popik et al., 1991b.)

of juveniles may be an important mediator of the olfactory cues.

The modulatory effects of drugs on learning and memory processes can be studied in many ways. Since the facilitatory effects of vasopressin and related peptides on learning and memory was demonstrated typically using passive or active avoidance paradigms, it was of interest to investigate these effects using another approach. The assessment of learning or memory cannot be measured directly but can be deducted by observing the effects of learning as expressed in the performance of an organism. However, there are many factors that may affect the performance of an organism in a learning situation that may not affect the learning process itself. Thus, to make a valid conclusion that an increase or decrease in learning performance of an animal reflects an improvement or impairment in the ability to learn, the possibility that the effects are due to performance factors should be ruled out. It seems that factors causing mental retardation in humans do not affect the rate of 'directed' learning, that is, learning in situations where the attention of the organism is directed by the experimenter to the stimuli relevant for the solution of a problem. Conversely, basic kind of learning takes place naturally in situations where learning is neither demanded nor directed by the experimenter and where it is without physiological motivation. This question of whether necessity is required for learning to occur is a very old one in psychology. Early proponents of the argument insisted that need or drive was necessary for learning. This line of argument directed the interest of human theorists away from any serious consideration of the animal as a model for human cognitive processing, since it was clear from human experience that no physiological need was necessary for human learning to occur. The problem of demonstrating learning without physiologically motivating animals is quite difficult, yet several experimental paradigms were created. The best known is that of tests of latent learning (Levitsky, 1979).

It seems that the recently developed social recognition test will be ideal for studying the effects of neurohypophyseal hormones on learning and memory. This assumption is based on the observation that social recognition test measures incidental learning which, similarly to other paradigms based on measuring incidental learning may be advantageous due to their sensitivity to the kinds of biological insults, which cause mental retardation in humans, including neonatal hypothyroidism, malnutrition or early phenylketonuria (Levitsky and Barnes, 1972; Levitsky and Strupp, 1981; Strupp et al., 1984).

Effects of neurohypophyseal hormones

In the 1960s De Wied found that removal of the posterior lobe of the pituitary in rats interfered with the maintenance of shuttle box avoidance behavior, and that this deficit could be restored by the treatment with pitressin or purified [Lys^8]vasopressin (De Wied, 1965). These and other findings resulted in the hypothesis that neurohypophyseal principles are implicated in memory processes. This hypothesis was supported by showing that peptides derived mainly from vasopressin enhance memory processes using different behavioral paradigms (for review see Van Ree et al., 1978; De Wied et al., 1993).

Learning consists of *acquisition* of new information. Subsequently, this information can be stored (*consolidated*), and can be used in particular situations (*retrieved*). Consolidation is a phase of this process taking place for several hours after acquisition, depending on the information acquired and the species (McGaugh, 1966; Squire, 1986). Treatments given just after learning will probably influence consolidation processes, whereas those given just before the retention test may affect retrieval processes. Soon after the studies on posterior lobectomized rats, it appeared that pitressin and synthetic vasopressin also affect *acquisition* and extinction of active and passive avoidance behavior in intact rats (De Wied and Bohus, 1966; De Wied, 1971; Ader and De Wied, 1972). A single injection of vasopressin resulted in a long-term, dose-dependent inhibition of the extinction of active avoidance behavior. The effects were still seen after the administered peptide had disappeared from the body (De Wied, 1971; De Wied et al., 1976). Furthermore, increased resistance to extinction of conditioned taste aversion has been shown (Rigter and Popping,

1976; Ettenberg et al., 1983b). Effects of vasopressin on *acquisition* of avoidance behavior can be demonstrated only under certain conditions, when the learning processes are attenuated, as is the case in hypophysectomized rats (De Wied, 1969; Lande et al., 1971; Bohus et al., 1973), or when the tendency to respond is low (King and De Wied, 1974). Thus, the acquisition of shuttle box avoidance behavior of hypophysectomized rats improved after treatment with vasopressin, while the peptide generally did not affect acquisition of avoidance behavior in intact rats. It has been shown that desglycinamide [Arg^8]vasopressin (DGVP) given subcutaneously 1 h before each of the six daily sessions facilitated learning and 'reference' and 'working' memories of rats in an appetitively motivated (hole board food search) task (Gaffori et al., 1985; Vawter and Van Ree, 1995). Buresova and Skopkova (1982) found an improved behavioral performance following administration of vasopressin-derived peptides in a 24-arm radial maze in which the behavior was reinforced by food. Another vasopressin-derived peptide (DDVP) facilitated acquisition and impaired reversal learning of a brightness discrimination, a food reinforced task (Couk and Beckwith, 1982). The same substance had no significant effect on the accuracy of delayed matching behavior in the pigeon, an animal model of short-term memory (Teal and Evans, 1982). Vasopressin-like peptides also facilitated acquisition of an appetitive task such as alcohol drinking behavior (Finkelberg et al., 1978). Some studies on vasopressin and related peptides favored the hypothesis that these principles facilitate memory *consolidation* of avoidance learning. Activity of these peptides occurred when injected just after training and 1 but not 3 or 6 h later (De Wied, 1971; Bohus et al., 1972). In a appetitively motivated task, vasopressin given just after the learning trial was found to potentiate the learned response (Ettenberg et al., 1983a,b). Vasopressin enhanced a one-trial passive avoidance task to suppress spontaneous pecking behavior of the chicks (Davis et al., 1982). Vasotocin has been shown to delay extinction of a conditioned avoidance behavior in neonatal chicks when given just after training (Davis and Pico, 1984). To the contrary, oxytocin facilitated *extinction* of a bench-jumping active avoidance response (Schulz et al., 1974) and attenuated passive avoidance behavior in rats (Kovacs et al., 1978). Vasopressin and related peptides were shown to affect *retrieval* processes in rats as well. Treatment with the peptide prevented or reversed the retrograde amnesia (for a passive avoidance response), induced by CO_2 inhalation (Rigter et al., 1974) or by electroconvulsive shock (Pfeifer and Bookin, 1978). Similar effects were exerted on pentylenetetrazol-evoked amnesia (Bohus et al., 1982), puromycin-induced retrograde amnesia for a maze learning task in mice (Lande et al., 1972), and retrograde amnesia induced by circadian disruption (Fekete et al., 1986). Vasopressin has been found to reactivate memory after hypothermia-induced amnesia (Tinius et al., 1985). In an appetitively motivated black-white discrimination T-maze, animals demonstrated prolonged extinction if received vasopressin (pitressin) before daily extinction sessions (Hostetter et al., 1977). Treatments attenuating and/or producing amnesia are regarded as influencing retrieval processes (Spear, 1973). Since oxytocin attenuated passive avoidance behavior when given just before the retention session (Bohus et al., 1978a), both hormones seem to affect memory retrieval in an opposite manner.

Vasopressin given peripherally after initial training, was shown to facilitate social recognition of rats (Dantzer et al., 1987). In the same study, attenuation of social recognition by oxytocin has also been reported (Dantzer et al., 1987). The attenuation of social recognition by oxytocin was confirmed in our studies that in addition indicated that an oxytocin antagonists not only reversed the attenuation of social recognition by oxytocin, but also appeared to facilitate social recognition on their own (Popik and Vetulani, 1991). Further studies with oxytocin indicated that this neurohypophyseal hormone *facilitated* social recognition at much lower (ng/kg) doses given peripherally (Popik et al., 1992a) (see Fig. 3). These findings were later confirmed by Benelli et al. (1995).

Vasopressin (1.5 μg per rat) facilitated social recognition both of intact juveniles as well as of juveniles that were washed (that is devoid of body scents). Vasopressin, however was ineffective in facilitating social recognition of preputialecto-

mized juveniles, indicating that the preputial glands are the source of odors that are crucial for social recognition to occur (Popik et al., 1991a). Facilitation of social recognition was found also for vasopressin used at the dose of 3 μg/kg (Popik et al., 1991b). In the same studies it appeared that social recognition depends on the decrease of anogenital exploration (Fig. 1), that is one of the several social activities occurring during social encounters (Popik et al., 1991b). These data suggest that the recognition of olfactory signals produced by anogenital areas allow rats to recognize each other.

Another paradigm based on odor and recognition is the so-called social transmission of food preferences (Bunsey and Strupp, 1990; Strupp et al., 1990). The effects of a vasopressin related peptide (DGVP) and oxytocin were investigated in a similar test procedure employing flavored tea rather than flavored food as a distinct cue (Popik and Van Ree, 1993). It was found that both DGVP (15 μg/kg) as well as oxytocin in a wide range of doses facilitated the memory for the presented new flavor (Fig. 4). The effect was stimulus specific.

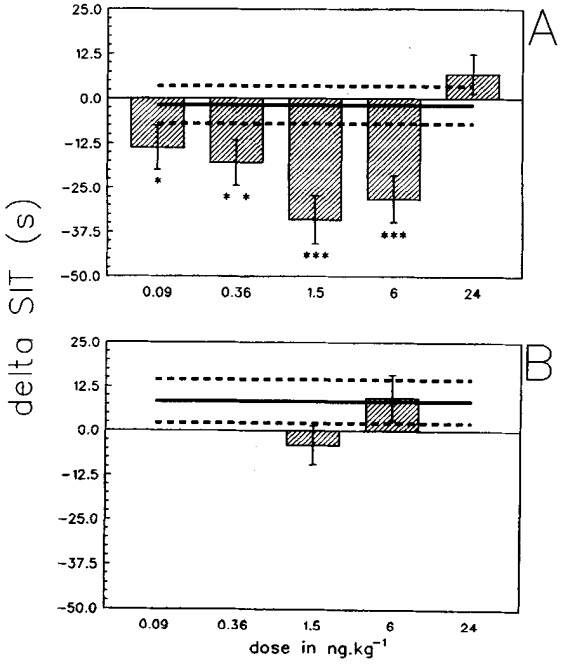

Fig. 3. Low doses of oxytocin facilitate social recognition. Presented are the difference in social investigation time (SIT) during the first and second encounter (Δ SIT; second SIT-first SIT; a lower Δ means a better memory). The encounters with the same (A) or a different (B) juvenile were separated by 120 min, i.e. the interval after which placebo-injected subjects (solid line [mean] and dashed lines [SEM]) were unable to recognize the juvenile. Oxytocin (0.09–24 ng/kg) was subcutaneously administered just after the first encounter. Asterisks indicate a difference from the first encounter (*$P < 0.05$, **$P < 0.025$, ***$P < 0.001$). (From Popik et al., 1992a.)

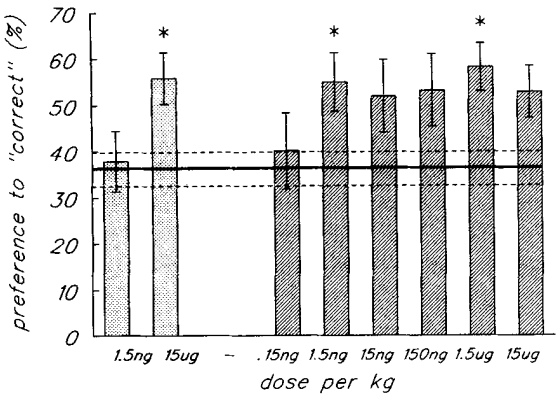

Fig. 4. Effects of DGVP and oxytocin on social transmission of flavored solution preference. The 'Demonstrator' rat had consumed a flavored solution before an encounter with an 'Observer' rat. Two hours after the encounter the Observer was given a choice between the solution consumed by the Demonstrator and a new flavored solution. Preference for the solution consumed by the Demonstrator indicates memory for that flavor (and the encounter). Placebo-injected Observers (solid line [mean] and dashed lines [SEM]) preferred the 'correct' solution less than Observers injected subcutaneously with different doses of DGVP (open bars) or oxytocin (hatched bars). *Different from placebo-treated animals (*$P < 0.05$). (From Popik and Van Ree, 1993.)

Central versus peripheral action

The question of central or peripheral site of action of neurohypophyseal hormones on learning and memory was investigated following at least two procedures, i.e. intracerebral administration and the use of peptides devoid of peripheral effects. The later experiments involved structure-activity studies. It should be noted that the central site of action of neurohypophyseal peptides on learning

and memory processes was already likely considering the comparison of behavioral and hormonal actions of vasopressin and oxytocin. For example, oxytocin shares with vasopressin its pressor and antidiuretic actions, although it is less potent. In contrast, as mentioned earlier, the two hormones have opposite effects on learning and memory, hence suggesting that the peripheral mechanism of action can hardly explain the memory effects.

In general, peptides pass the blood-brain barrier with difficulties (Zaidi and Heller, 1974). However, there are indications that vasopressin and oxytocin exert their effects by acting on the brain. When given intracerebroventricularly in doses of about three orders of magnitude lower than after peripheral administration, they induced similar behavioral effects as described for subcutaneous administration (Bohus et al., 1978a,b; Koob et al., 1981). Almost the same, three orders of magnitude lower concentrations of neurohypophyseal peptides have been found in the cerebrospinal fluid after peripheral administration (Mens et al., 1983).

Le Moal et al., (1987) found in adult male rats that vasopressin injected intracerebroventricularly at doses of 0.5–2.0 ng immediately after investigation of the juvenile, decreased social investigation of the same juvenile at the long (120 min) interexposure interval (IEI). This decrease in investigatory time was similar to that observed after a 30-min IEI in untreated animals. The same effects were reported when vasopressin was administered chronically by the intracerebroventricular route (Bluthé and Dantzer, 1992). Benelli et al. (1995) found that oxytocin in a dose range of 1 fg–10 ng/rat intracerebroventricularly, immediately after a 5-min encounter with a juvenile, facilitated social recognition and that an oxytocin antagonist blocked that effect.

Structure-activity studies

The effects of neurohypophyseal hormones on learning and memory processes may be due to their peripheral effects. The 'classical endocrine' action of vasopressin has generated some hypotheses about the mechanism of action with respect to its memory enhancement in the avoidance studies. Since peripheral injection of vasopressin in pharmacological doses results in unpleasant sensations, perhaps due to the increase in blood pressure (Altura, 1967; Matsuguchi et al., 1982) and its dipsogenic action (Szczepanska-Sadowska et al., 1982), it was postulated that this interoceptive cue might act as additional negative reinforcement during avoidance learning (Koob, 1987), which is negatively reinforced by itself (foot shock) (Ader et al., 1972). It has been shown convincingly that peripheral administration of vasopressin is aversive to rats (Ettenberg et al., 1983b; Ettenberg, 1984). Injections given around acquisition may thus enhance the aversiveness of the training and therefore delay extinction (Ettenberg et al., 1983b) in the same way as does raising the shock intensity in avoidance studies (Ader et al., 1972). However, the same effects on memory processes were obtained after injection of vasopressin fragments being synthesized either in the laboratory or by brain enzymes that lacked the 'classical endocrine' action of the parent molecule, hence making an 'aversive' explanation of mnemonic effects, as proposed by Ettenberg et al. (1983a) unlikely. It seems that for the 'classical endocrine' actions, the whole intact molecule is necessary. Removal of either one or a few amino acid residues from the parent molecule causes a loss of the endocrine activity, but the behavioral actions can be retained (for review see Van Ree et al., 1978). Removal of the C-terminal amino acid glycinamide residue yielded a peptide, DGVP, with a somewhat (about 50%) reduced behavioral potency, but without apparent endocrine activity (Lande et al., 1971; De Wied et al., 1972). Desglycinamide-vasopressin prevented the amnesia caused by puromycin in mice (Lande et al., 1972) and slowed extinction of pole jumping avoidance behavior (Walter et al., 1975).

Experiments with various fragments of vasopressin have revealed that the memory enhancing activity is located in the covalent ring structure, although a second active site for this effect might be present in the linear part of the molecule (De Wied, 1976). Effects of neuropeptides on consolidation processes can be elaborated by studying passive avoidance behavior. Vasopressin, DGVP, the covalent ring structure of vasopressin and surprisingly also oxytocin, enhanced the resistance to extinction,

given intracerebroventricularly just after the learning trial. Although post-learning administration of the linear parts of both neurohypophyseal hormones also facilitated passive avoidance behavior, these fragments were less active (Kovacs et al., 1981). The C-terminal linear parts of the vasopressin and oxytocin molecules, in contrast to their covalent ring structures, were active in protecting against experimentally induced amnesia (Walter et al., 1975). Since attenuation of retrograde amnesia is regarded as an action on retrieval processes (Spear, 1973), it was hypothesized that, in particular, the C-terminal linear part of the neurohypophyseal hormones contains the sequence involved in memory retrieval (Van Ree et al., 1978).

The structure activity studies corroborated the view that fragments of neurohypophyseal hormones are responsible for the central nervous system effects, and hence, it was postulated that neurohypophyseal hormones are precursor molecules of more potent and selective neuropeptides involved in the consolidation and retrieval of memory processes (De Wied and Bohus, 1978). Support for this idea came from studies on the fragments generated from vasopressin and oxytocin by brain synaptic membranes in vitro (Burbach et al., 1983b; De Wied et al., 1984). It was found that some of these fragments (particularly [pGlu^4,-Cyt^6]VP 4–8 and [pGlu^4,Cyt^6]VP 4–9) are highly potent and selective memory enhancing neuropeptides (Burbach et al., 1983b; De Wied et al., 1987; Bunsey et al., 1990). Interestingly, also oxytocin fragments devoid of peripheral endocrine activity of oxytocin like [pGlu^4,Cyt^6]OT 4–8 and [pGlu^4,-Cyt^6]OT 4–9 more potently attenuate memory processes than the whole oxytocin molecule (Burbach et al., 1983a; De Wied et al., 1987, 1991).

Structure-activity studies with both vasopressin and oxytocin-related peptides were also performed using the social recognition paradigm. Rats that were unable to recognize juveniles after 60 or 120 minutes demonstrated social recognition when treated with desglycinamide[Arg^8]vasopressin (DGVP) in the dose of 6 μg/kg or [pGlu^4,Cyt^6]VP 4–8 (VP 4–8) used in the dose of 1 μg/kg (Popik et al., 1991b). Since these peptides are devoid of the peripheral endocrine actions, these data suggest that the facilitatory effects of vasopressin and vasopressin-related peptides on social recognition are centrally mediated and do not involve pressor or dipsogenic effects of vasopressin. The effects of vasopressin-related peptides on social recognition was further characterized in studies of Sekiguchi et al. (1991b). They found that vasopressin fragments, including VP 4–8, VP 4–9, VP 5–8 and VP 5–9 facilitated social recognition. Moreover, the facilitatory effects of DGVP appeared to be dose-dependent in that doses lower than 6 μg/kg of that peptide appeared inactive. In addition, DGVP allowed rats to recognize juveniles from 2 to 24 h, but not 1 or 48 h after the initial encounter, suggesting that there is specific time window for the social recognition to occur. In other studies, we found that desglycinamide vasopressin [VP-(1–8)] as well as VP-(1–7) or VP-(1–6) facilitated social recognition up to 24 h after the first encounter (Popik and Van Ree, 1992). Structure-activity data suggest that this long-term facilitation of social recognition depends upon the covalent ring structure of vasopressin.

In subsequent studies (Popik et al., 1996), we investigated the modulating effects of oxytocin [OT 1–9], desglycinamide-oxytocin [OT 1–8], tocinamide (OT 1–6, TOC), Pro-Leu-Gly (OT 7–9, PLG), Leu-Gly (OT 8–9, LG), [pGlu^4,Cyt^6]OT 4–8 [OT 4–8], [pGlu^4,Cyt^6]OT 4–9 [OT 4–9] and glycine on social recognition. Recognition-facilitating effects were studied by giving each peptide in the doses of 0.6 and 6.0 ng/kg subcutaneously just after the first encounter, using the long (120 min) IEI. Recognition-attenuating effects were studied by giving 0.6 and 6.0 μg/kg subcutaneously of each peptide just after the first encounter, using the short (30 min) IEI. It was found that at low doses, only OT 1–9, PLG and LG facilitated social recognition. In contrast, high doses of all the peptides studied, except OT 8–9, attenuated social recognition. Particularly effective were OT 1–9, OT 1–8, OT 4–9 and OT 7–9. The results of these studies support the hypothesis that oxytocin related peptides play a dual role in social recognition and that attenuation and facilitation of social memory are mediated by different mechanisms and/or other parts of the oxytocin molecule. The facilitation of social memory may reside in the C-terminal part of oxytocin, while the attenuation may reside in the 5–7 region of oxytocin molecule (Popik et al., 1996).

Site of action in the brain

An important question for agents exerting effects on learning and memory is about their site of action within the brain. If damage of a particular brain area is followed by an impairment of learning and/or memory, this area may be considered to be critical for learning and/or memory processes. Substances active when given to those structures may be regarded as memory modulators (Koob et al., 1989).

Some restricted brain areas (in particular of the limbic system) including the dorsal hippocampus, septum, amygdala and parafascicular nucleus are sensitive for the memory-modulating effects of vasopressin and oxytocin as assessed with passive avoidance behavior (Kovacs et al., 1979). Electrolytic lesions in the septal complex or the dorsal hippocampus prevented the effects of vasopressin on the maintenance of an active avoidance response (Van Wimersma Greidanus and De Wied et al., 1976). Microinjection of minute amounts of vasopressin into the hippocampal dentate gyrus or into the dorsal raphe nucleus immediately after the learning trial facilitated the retention of a passive avoidance response (Kovacs et al., 1979). The same amounts of oxytocin exerted an opposite effect; passive avoidance behavior was attenuated. Vasopressin and oxytocin were also effective when injected into the dorsal septal area but here the opposite effect on consolidation was not present; both peptides facilitated the retention of the passive avoidance response. Vasopressin given to the hippocampal dental gyrus and central amygdaloid nuclei reversed pentylenetetrazol-induced amnesia, indicating effects on memory retrieval, (for review see Bohus et al., 1982). The brain areas sensitive for memory modulating action of neurohypophyseal hormones correspond well with the projections of extra-hypothalamic vasopressin and/or oxytocin containing nerve terminals (Buijs et al., 1980). The septum appeared to be a sensitive site for vasopressin in facilitating social memory (Dantzer et al., 1988; Popik et al., 1992b; Engelmann and Landgraf, 1994; Everts and Koolhaas, 1997). A similar, facilitatory action of vasopressin and oxytocin administered into the septal area, as described for avoidance learning (Van Ree et al., 1978; De Wied and Bohus, 1979) was found in social recognition paradigm. Thus, picogram doses of vasopressin, [pGlu^4,Cyt^6]VP 4–8 and oxytocin given intraseptally produced facilitation of social recognition (Popik et al., 1992b). The effects of vasopressin were blocked by V_1 and V_2 receptor antagonists, but not by an oxytocin receptor antagonist. Interestingly, the facilitatory effects of intra-septal oxytocin was not blocked by the vasopressin or oxytocin antagonists.

Blocking the V_1 vasopressin receptor in the septum with an antisense oligonucleotide attenuated social recognition and the vasopressin action on social recognition (Landgraf et al., 1995). Inactivation of vasopressin by injecting anti-vasopressin serum into the dorsal septum or in the dorsal or ventral hippocampus, but not in the nucleus olfactorius attenuated social recognition. Local administration of anti-oxytocin serum in the ventral hippocampus, but not in the dorsal hippocampus, nor in the nucleus olfactorius or the septal area facilitated social recognition. Thus, the presence or local release of vasopressin and oxytocin in the ventral hippocampus and that of vasopressin in the dorsal hippocampus and dorsal septal area are of physiological importance for social recognition (Van Wimersma Greidanus and Maigret, 1996).

Another brain area sensitive to the modulatory action of neurohypophyseal peptides on social recognition is the medial preoptic area. Our studies have revealed that picogram amounts of oxytocin but not vasopressin, [pGlu^4,Cyt^6]VP 4–8 or [pGlu^4,Cyt^6]VP 4–9 facilitated social recognition when administered into that brain area (Popik and Van Ree, 1991, see Fig. 5). The sensitivity of the medial preoptic area of hypothalamus to the facilitatory effects of oxytocin may suggest that social recognition depends also on other mechanisms, not necessarily *directly* related to learning or memory. The medial preoptic area plays a critical role in regulating masculine sexual behavior (Edwards and Einhorn, 1986; Kondo et al., 1990), stereotypical behaviors related to the establishment of the dominance hierarchy (Ferris et al., 1984) and processing of olfactory information (Pfaff and Pfaffmann, 1969; Macrides, 1976). Moreover, the synthesis of vasopressin is under control of gonadal steroids and, more importantly, castration modifies

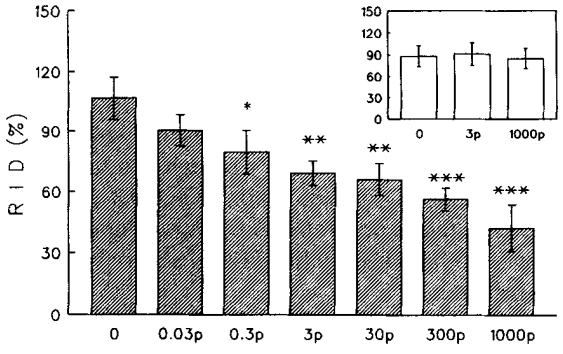

Fig. 5. Oxytocin facilitates social recognition after administration into the medial preoptic area. Presented are means and SEM of ratios of investigation duration (RID) (the lower RID, the better memory). The encounters with the same (main panel) or a different (inset) juvenile were separated by 120 min, an interval after which placebo-injected subjects are unable to recognize the juvenile. Graded doses of oxytocin (0.03–1.000 pg) or placebo (0) were administered into the medial preoptic area just after the first encounter. *Different from placebo treatment (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (From Popik and Van Ree, 1991.)

the ability of male rodents to recognize a social object (Bluthé et al., 1990, 1993; Bluthé and Dantzer, 1990). Thus, the effects of neurohypophyseal peptides administered into the medial preoptic area may be difficult to interpret in terms of specific effects on learning and memory processes.

Concluding remarks

Our studies on the effects of neurohypophyseal hormones on social recognition focused on: (1) the ethological evaluation of the social recognition phenomenon, (2) the effects of peripheral administration of vasopressin, oxytocin and related peptides on social recognition, (3) the effects of local administration of vasopressin and oxytocin into the septal and medial preoptic areas of the rat's brain and (4) the action of vasopressin and oxytocin on another socially mediated kind of learning, social transmission of information.

We found that the cue used by rats in the social recognition paradigm is an odor of the juvenile conspecific, most probably produced by the preputial glands. Accordingly, anogenital sniffing is the most prominent rat's activity during encounters, being mainly responsible for the decrease of social investigation on the second encounter. Peripheral treatment with vasopressin and vasopressin related peptides facilitated social recognition, as did the treatment with oxytocin antagonists. Oxytocin attenuated social recognition after peripheral administration. However, oxytocin given systematically in low doses facilitated recognition of the juvenile. Vasopressin and related peptides exerted long-term effects on social memory, a phenomenon thought to reflect a short-term memory processes in drug-free rats. Structure-activity studies revealed that an intact ring structure of vasopressin is needed for this long-term effect. Structure-activity studies with oxytocin and related peptides demonstrated that at low doses, only oxytocin, and the C-terminal fragments, OT 7–9 (PLG) and OT 8–9 (LG) facilitated social recognition. In contrast, high doses of most oxytocin peptides studied attenuated social recognition. Particularly effective were oxytocin and the oxytocin fragments OT 1–8, OT 7–9 and OT 4–9. The septum appeared to be a sensitive brain site for the social recognition facilitatory action of vasopressin, the vasopressin related peptide (VP 4–8) and oxytocin, whereas vasopressin had no effect when injected into the medial preoptic area of the rat brain. However, oxytocin administration in this brain area facilitated social recognition with the dose as low as 0.3 pg being effective. The vasopressin related peptide, (DGVP) as well as oxytocin facilitated social transmission of information between rats. Also in this paradigm low doses of oxytocin appeared to be effective.

Social recognition as a test model for learning and memory processes

There are several distinct arguments to characterize the social recognition paradigm as a test measuring short-term, working memory.

(1) Social recognition decreases significantly as a negative function of the time between initial and subsequent encounter (Thor and Holloway, 1982; Popik et al., 1991b; see Fig. 2). This phenomenon

suggests that, as is the case in other memory paradigms, the maintenance of the information about the conspecific is kept to certain time limits, depending probably on the time course of forgetting.

(2) The decrease of social behavior in the paradigm is not due to habituation or fatigue, because: (a) it does not occur when novel juveniles are presented, and (b) the quantity of other social activities, not related to social recognition, remains relatively unchanged (Popik et al., 1991b). The same conclusion was reached by Hlinak and Krejci (1991) who found that whereas social sniffing decreases in case of drugged rats, sniffing of scent traces left on the floor of the test boxes does not differ between experimental and control animals. That means also that when a priori defined recognition occurs, the decrease in social behavior displayed by residents concerns only certain activities, in particular social sniffing (see also Popik et al., 1991b).

(3) According to the present knowledge about learning processes, the time after which acquired information is used, depends to a certain extend upon the time of learning. Although in experiments conducted by Sekiguchi et al. (1991a), prolongation of the first exposure over 300 s had no effect on the time after which the recognition occurs, *shortening* of the first exposure session influenced that time (unpublished data). It was found that after an inter-exposure interval (IEI) of 20 min, the social investigation time was decreased in both vasopressin and placebo treated residents, when the encounter lasted 300 sec. When this encounter lasted 150 s, only vasopressin-treated rats diminished their social activity. Independently of the treatment, no social recognition was found when the encounter lasted 60 s (Popik, 1991). These findings suggest that also in the social recognition paradigm, the length of the maintenance of information concerning the juvenile, depends among others, on the length of the learning time (acquisition trial).

(4) The demonstration of retroactive manipulations is a classical procedure in research on memory. It is thought that the memory trace is maintained by a subject between the acquisition trial and the 'test'. Laboratory manipulations that specifically interfere with this trace modulate the strength of previously acquired information. Nonspecific manipulations should not influence the strength of the trace (Heise, 1984). Accordingly, it has been found that social recognition is sensitive for retroactive inhibition, by demonstrating that the presentation of another juvenile to the resident rat between the first and second exposure increased the social investigation time toward the initially encountered subject, thus decreasing social recognition (Thor and Holloway, 1982; Dantzer et al., 1987). Moreover, Dantzer et al. (1987) have shown that social recognition is also sensitive to retroactive facilitation, since an additional exposure to the initially encountered juvenile, enabled the residents to recognize it after a significantly longer time of IEI.

(5) The sensitivity of a test for retroactive inhibition requires that the interfering stimuli are *specific*. This has been confirmed by Perio et al. (1989) who found that removing the resident rat from his home cage just after the first encounter did not diminish subsequent recognition of a just encountered juvenile. Also we have found, that the resident rat recognizes a (singly housed) juvenile in the juvenile's home cage (unpublished data). Additionally, Hlinak and Krejci (1991) performed experiments on social recognition in an unfamiliar experimental box, which was common to all residents tested. Hence, it seems that non-specific stimuli do not result in retroactive inhibition.

(6) As mentioned earlier, the time after which recognition of the same juvenile occurs, is limited to about 30–40 min after the initial encounter. Under conditions of retroactive facilitation it could be prolonged to 120 min. Since peptide treatment enables the residents to recognize the formerly met juvenile after an IEI of 24 h (Sekiguchi et al., 1991a,b; Popik and Van Ree, 1992), the question arises whether it is possible to find such an effect in drug-free animals under conditions of retroactive facilitation. Sekiguchi et al. (1991a) were however unable to prolong the time after which recognition occurs to 24 h, by exposing residents to the same juvenile repeatedly, which may suggest that social recognition could not be manifested after such long time even in retroactive facilitation conditions.

(7) The motivation for, and the reinforcement in

social learning are obscure. It could be that the encounter with a conspecific per se is rewarding in nature, as social isolation has been frequently regarded as a punishment. Thus, the behavior of resident rats may be considered as a consequence of an innate need for investigation (Barnett, 1967), and the motivation for social memory may be 'internal', similarly as in the other socially mediated kinds of memory (Strupp and Weingartner, 1984).

(8) If one agrees that social recognition reflects a memory for the juvenile conspecific, the nature of this memory could be addressed. Is this memory a kind of a short or a long-term memory? From the experiments on retroactive inhibition described in this section, it appears that residents are unable to store information about more than one juvenile at the same time, indicating features characteristic for short-term, i.e. capacity-limited memory (Squire, 1986). Moreover, among drug-free residents, the recognition is not present after an IEI of 30 min, also suggesting a short-term process. However, some of the vasopressin-related peptides can extend this memory up to 24 h (Sekiguchi et al., 1991b; Popik and Van Ree, 1992). It could be that vasopressin-related peptides affects mechanisms dealing with transcription of information from the short to the long-term storage. On the other hand, some kind of social recognition may exist among drug-free rats even after 3 months of IEI. Although the amount of anogenital sniffing was similar for residents that encountered the same or different juveniles 3 months earlier, less aggressive behaviors were observed among residents that encountered the same juveniles (unpublished data).

(9) Every experimental procedure dealing with investigation of memory processes has working and reference memory components. Working memory concerns information obtained during a trial that is useful for that trial *only*. Reference memory concerns information obtained during a trial that is useful for *all* further trials. It is believed that all behavioral tasks have a reference memory component. The only question is whether or not there is also a working memory component (Olton et al., 1980). It seems that the social recognition paradigm concerns mainly working memory components, since the only relevant information is that dealing with the identity of the juvenile conspecific met on the first encounter. This information has to be used on the subsequent exposure and seems to be most important to the rat. However, in the social recognition paradigm reference memory components are also inevitably of importance, since the residents perform better from trial to trial, during the first few days of the experiment (Dantzer et al., 1987).

(10) Another aspect of behavioral tasks concerns distinction between intentional (directed) and incidental learning, bearing relevance for effortful and automatic cognitive operations (see also the section on Social memory paradigms). Intentional and incidental learning processes can be affected independently, since, for example, various manipulations that are thought to heighten arousal seem to impair incidental but enhance or have no effect on intentional learning. Although the incidental-intentional learning dichotomy concerns the type of information that is processed, it addresses the distinction between automatic and effortful learning, which, in turn, involves distinct neurobiological systems (Strupp and Weingartner, 1984). Thus, while for the effortful processing of information and intentional learning, the attention of the subject is crucial, automatic processing of information and incidental learning involves passive, 'shadowing' of information that requires relatively little cognitive capacity. In the social recognition paradigm an incidental rather than an intentional type of learning may be of the relevance.

In conclusion, the decrease in social investigation time on repeated encounters can be attributed to social recognition, which reflects social memory. It seems that in the social memory paradigm, short-term, working memory is investigated. It is likely that this memory is formed as a consequence of internally motivated, incidental learning.

The social recognition paradigm can be used to investigate effects of drugs on memory processes. For example, Perio et al. (1989) found that several drugs, besides vasopressin and oxytocin neuropeptides that affect learning and memory processes are effective in the social recognition paradigm. These include arecoline, physostigmine, nicotine, aniracetam and beta-carbolines. Conversely, drugs appearing as 'false positives' in other tests aimed at

measuring memory facilitation, including amphetamine, nomifensine and strychnine were ineffective in the social recognition paradigm or reduced social behavior non-specifically. Others found similar facilitatory effects on social recognition for glutamatergic antagonists and nootropic drugs (Lederer et al., 1993; Hlinak and Krejci, 1994, 1995). In a recent study, Terranova et al. (1996) found that social recognition deficit produced by intra-septal administration of amyloid beta-peptides can be prevented by chronic peripheral administration with the non-peptide neurotrophic compound SR 57746A. Social recognition paradigm has also been used to investigate the facilitatory effects of histamine and related compounds (Prast et al., 1996), the reversible acetylcholinesterase inhibitor, P10358 (Smith et al., 1997), and a mixed 5-HT_4 agonist/5-HT_3 antagonist, BIMU1 (Letty et al., 1997). The treatment with alcohol during development (days 2–10 after birth) resulted in the attenuation of social recognition in adulthood (Kelly and Tran, 1997).

Memory effects of vasopressin and oxytocin as revealed by social recognition

Squire and Davis (1981) have listed criteria useful in considering whether a particular brain system is critically involved in memory modulation. These criteria will be described in relation to the existing data concerning neurohypophyseal hormones and social recognition.

(A) *Effects of drugs affecting the brain system should be time and dose dependent.* Though the oxytocin-induced attenuation of social recognition resembled rather an on/off phenomenon (Popik and Vetulani, 1991), in general, the effects of neurohypophyseal hormones on social recognition follow this postulate. Thus the peripheral treatment with DGVP and [pGlu^4,Cyt^6]VP 4–8 (Sekiguchi et al., 1991b) as well as treatment with low doses of oxytocin (Popik et al., 1992a) have been found to facilitate social recognition in a dose-dependent fashion. The same has been found for oxytocin given into the septal and medial preoptic areas and for vasopressin given into the septum (Popik and Van Ree, 1991; Popik et al., 1992b). Also the findings of Sekiguchi et al. (1991b) demonstrate that the effects of DGVP administered peripherally are dose-dependent. In addition, these authors found that rats injected with DGVP are able to recognize the juvenile conspecific 2–24 h, but not 48 h after the first encounter, suggesting a time window of the facilitating action of this peptide on social memory. Data concerning time-dependent effects (i.e. aimed at investigating the sensitive interval between the learning trial and drug administration) of treatment with neurohypophyseal hormones on social recognition are lacking.

(B) *Facilitation as well as disruption of memory should occur with appropriate manipulation of the system.* Both subcutaneous and intra-septal administration of vasopressin and vasopressin-derived peptides facilitated social recognition (Popik et al., 1991b, 1992b). The effects of either peripheral (Dantzer et al., 1987) or septal injections (Popik et al., 1992b) could be prevented by the pre-treatment with vasopressin antagonist(s) that exerted an own, opposite effect on social recognition. Oxytocin given peripherally attenuated social recognition (Dantzer et al., 1987; Popik and Vetulani, 1991), and this action could be antagonized by oxytocin antagonists that induced an own, facilitating effect on social recognition (Popik and Vetulani, 1991).

(C) *Removal of the system should affect memory.* The septal (Dantzer et al., 1988; Popik et al., 1992b; Engelmann and Landgraf, 1994) and medial preoptic (Popik and Van Ree, 1991) areas are sensitive for the vasopressin and oxytocin facilitating action respectively on social recognition. Although there are no data available showing that lesions of the septal or medial preoptic area influence social recognition, it has been demonstrated that castration disrupted temporary social recognition (Bluthé et al., 1990, 1993). The relevance of this finding is supported by the fact that vasopressinergic innervation of the septum is androgen-dependent. Castrated rats were insensitive for the attenuating action of a vasopressin V_1 receptor antagonist. These data may suggest that when the androgen-dependent vasopressin innervation is not functioning, the social recognition is impaired. The administration of specific receptor antagonists may lead to a decrease of functioning of the system. Both vasopressin (Dantzer et al., 1987; Engelmann and Land-

graf, 1994) and oxytocin (Popik and Vetulani, 1991) antagonists have been found to exert opposite effects on social recognition than the neurohypophyseal hormones themselves. Recently, Maaswinkel et al. (1996) reported that fimbria-fornix (but not basolateral amygdala) lesioned rats demonstrated impaired social recognition.

(D) *There should be a correlation between learning and some measure of physiological or neurochemical activity of the system.* As far as the social recognition paradigm is concerned, this postulate can be neither confirmed nor denied. Some experiments suggest that the vasopressin level in the septum during the encounter with a juvenile remains unchanged, as measured by the push–pull technique (M. Engelmann, pers. commun.). This approach needs further investigation.

(E) *Effects of manipulating the system should be obtainable across a variety of tasks, not just aversive or appetitive tasks or tasks requiring movement.* This criterion is fulfilled by experiments discussed earlier. In addition to social recognition experiments, some observations done in another socially mediated learning task, i.e. socially mediated flavored tea preferences (Popik and Van Ree, 1993) seems to support the mnemonic role of neurohypophyseal hormones. It has been found that the vasopressin derivative (DGVP) and oxytocin may prolong the time, after which an 'observer' rat is able to maintain the information about the flavored tea solution consumed by a 'demonstrator' rat. The facilitating effects of DGVP and low doses of oxytocin correspond well with the similar effects revealed in social memory paradigm. In addition the time that the information was maintained was comparable in both paradigms.

In conclusion, the studies about social recognition and neurohypophyseal hormones support the postulate about the role of vasopressin and oxytocin in memory processes. Vasopressin, oxytocin and related peptides modulate social recognition and social transmission of information.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
Progress in Brain Research, Vol. 119

CHAPTER 5.4

Coping with stress in rats and mice: Differential peptidergic modulation of the amygdala–lateral septum complex

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This chapter focuses on the parvicellular vasopressin (VP) system originating from the medial nucleus of the amygdala (MeA) and bed nucleus of the stria terminalis (BNST). The vasopressinergic fibers of these nuclei innervate a number of limbic brain areas including the septum–hippocampal complex. Interestingly, this VP system is sexually dimorphic and the VP synthesis in this system depends on circulating gonadal steroids. Studies in rats and mice show that the variation in the lateral septal VP network within the male gender is as large as the variation between the sexes as reported in the literature. Non-aggressive males are characterized by a far more extensive VP network and a higher VP content in the lateral septal area than aggressive males. A review of the literature on the function of lateral septal VP in the organization of behavior reveals not only a modulatory role of behavior in a social context, but also of fear- and anxiety-related behaviors. It is argued that these seemingly diverse functions might be explained by the concept of coping style. Extensive behavioral and physiological analyses in a variety of animal species show that males may be characterized by the way in which they cope with environmental challenges in general. Aggressive males tend to cope actively with their environment whereas non-aggressive males seem to accept the situation as it is more easily. In several tests, we determined the effects of chronic infusion of the V_1 receptor antagonist locally into the lateral septal area in male rats. The main conclusion from these experiments is that LS VP does not modulate coping style in general. However, the experiments confirm the idea that LS VP has a certain degree of functional specificity in social behavior and social learning tasks. Together with the observation that the size and distribution of the vasopressinergic system may be highly variable between individual males in relation to their coping style, this suggests that the lateral septal vasopressinergic system is involved in the differential capacity of individuals to cope behaviorally with challenges of a social nature.

Introduction

The neuropeptide arginine vasopressin (VP) has a distinct distribution throughout the mammalian brain. VP containing cell bodies are not only found in the classical neuroendocrine structures such as the paraventricular nucleus and the supraoptic nucleus of the hypothalamus, but also in a number of extrahypothalamic brain structures. It is now clear that VP genes are expressed in neurons belonging to several VP subsystems in the brain, each with different projection areas and, presumably, with different functions. In rats, the VP system consists of two types of neurons: magnocellular and parvicellular (De Vries and Buijs, 1983; Sofroniew, 1983; De Vries et al., 1985). The magnocellular VP neurons are localized within the paraventricular hypothalamic nucleus (PVN), nucleus circularis, supraoptic nucleus (SON), and accessory nuclei. Parvicellular VP neurons are found in the hypothalamus, as well as in extrahypothalamic sites. They have been localized in the PVN (De Vries and Buijs, 1983; Sofroniew, 1983), the suprachiasmatic nucleus (SCN) (De Vries et al., 1981), the bed nucleus of the stria terminalis (BNST) (De Vries and Buijs, 1983; Caffé and Van Leeuwen, 1983),

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the medial nucleus of the amygdala (MeA) (Caffé et al., 1987; Caffé and Van Leeuwen, 1983). Recently, small VP expressing cells have been found in the horizontal diagonal band of Broca (Planas et al., 1995). Beside their different locations, the magnocellular and parvicellular type of neurons also have different projection areas. Magnocellular VP neurons project solely to the posterior pituitary from where the peptide is released into the general circulation. The parvicellular neurons of the PVN project both to the median eminence where the hormones are released into the portal vessel system to regulate anterior pituitary function, and to the brain-stem and spinal chord (for a review, see De Wied et al., 1993). The extrahypothalamic parvicellular VP neurons from the SCN project to hypothalamic areas (De Vries et al., 1981; Buijs, 1983), the MeA projects to the lateral septum and ventral hippocampus (Caffé et al., 1987; Metzger et al., 1993), whereas the BNST innervates mainly the olfactory tubercle, vertical diagonal band of Broca, lateral habenula, lateral septum, and the ventral hippocampus (De Vries and Buijs, 1983).

This chapter focuses on the parvicellular VP system originating from the MeA and BNST. The vasopressinergic fibers of these nuclei innervate a number of limbic brain areas including the septum–hippocampal complex. Interestingly, vasopressinergic information from the BNST and hippocampal information seem to converge at the same interneurons in the lateral septal area (Jakab et al., 1991). Vasopressinergic fibers from the BNST terminate at GABAergic spiny interneurons in the lateral septum which receive efferents from the hippocampus as well. In view of this convergence of input, one may expect that the lateral septum somehow integrates the influence of the amygdala and hippocampus in socio-emotional and spatial learning processes.

The purpose of the present chapter is to provide a possible conceptual framework and to summarize some of the supporting evidence for the integrative role of lateral septal VP in processes of an emotional and social nature. The evidence will be based on some of our studies on the individual differentiation in coping style in male rats and mice.

The BNST–LS vasopressinergic system

Gonadal hormone dependency

An important feature of the VP expression in the MeA and BNST neurons is that it is highly sensitive to circulating levels of gonadal hormones (De Vries et al., 1984, 1985; Albeck et al., 1991; Miller et al., 1992). When adult male rats are gonadectomized, a significant decrease in the VP fiber density occurs in the lateral septum, but also in areas such as the olfactory tubercle, diagonal band nucleus, basal nucleus of Meynert, ventral hippocampus, amygdaloid area, some paraventricular thalamic nuclei, lateral habenular nucleus, and the locus coeruleus. An almost complete disappearance of VP-ir after gonadectomy is accomplished in about 10 weeks. This is preceded by a significant decrease in the expression of cytoplasmic and nuclear VP mRNA in the BNST (and MeA) within 3 days. All these effects can be completely restored by replacement of testosterone (T); both VP and its mRNA reach intact levels again. Estrogen and the T metabolite dihydrotestosterone (DHT) are capable of partly restoring VP-ir. They seem to act synergistically to produce the full expression of VP (Miller et al., 1989a,b; Brot et al., 1993; Szot and Dorsa, 1993, 1994). Restoring the levels of lateral septal VP after gonadectomy by T replacement has been shown to be a time- and dose-dependent process. The maximum levels that can be reached correspond to the levels of intact animals. Increasing the T dose or period of treatment does not raise VP concentrations above that particular level. This suggest a regulatory mechanism that maintains lateral septal VP concentrations in the terminal regions at a certain set point (Magnusson and Meyerson, 1996).

Sexual dimorphism

An important characteristic of the VP-expressing cells in the BNST and MeA is their sexual dimorphism. Male rats have about two times as many VP-ir cells in the BNST as females. As a result, these cells provide a greater density of fibers projecting to the LS than is present in females (De Vries et al., 1981; Wang et al., 1993; Al-Shamma and De Vries, 1995). However, this sex difference does not

depend on differences in circulating hormone levels, since it persists in males and females that are treated with similar levels of T (De Vries and Al-Shamma, 1990). In addition to differences in cell number and fiber density, there are also sex differences in the receptors for gonadal steroids. As a result, the individual VP expressing cells in the BNST of males have a higher responsiveness to T, mediated through these androgen and estrogen receptors (De Vries et al., 1994; Lisciotto and Morrell, 1994; Wang and DeVries, 1995).

Although, at adult age, T is necessary for the integrity of BNST-LS projections, the sexual dimorphism is thought to be established by T at neonatal age. Both in male and in female rats, the first appearance of VP immunostaining in the LS occurs at postnatal day 10. At postnatal day 12, the sexual dimorphism becomes apparent. The development of VP mRNA expression is delayed in females compared to the males. As early as postnatal day 3, VP mRNA is detectable in the BNST in the male whereas in the female it cannot be found until day 14 (Szot and Dorsa, 1993; Snijdewint et al., 1989). Recently, it has been shown that T does so by acting on the estrogen receptor, which is mediating the initial expression of VP mRNA in the BNST of both sexes. The time difference in neonatal onset of VP expression is thought to be a factor explaining the sexual dimorphism in the VP system.

Individual differences within the male gender

Several studies indicate that the BNST-lateral septal vasopressinergic system is not only sexual dimorphic, but shows a large individual variation within the male gender as well. For example, when male Wistar rats are selected for their performance in a footshock motivated brightness discrimination task, the VP levels in the lateral septum in the high performance animals were significantly higher than in the low performance animals (Ermisch et al., 1986). Studies in lines of mice genetically selected for another emotional response, namely aggression, show a significant negative correlation between lateral septal VP and aggression (Compaan et al., 1992). The genetic selection was based on the attack latency during the confrontation of a resident male mouse with an unfamiliar male intruder in the home territory. Non-aggressive male mice are characterized by a more dense VP immunoreactive innervation of the LS and a higher number of VP neurons in the BNST compared to aggressive males. In addition, a radioimmunoassay for VP confirmed the findings that non-aggressive males have a higher lateral septal VP content compared to aggressive male mice. The VP content and the immunostaining in the aggressive males almost resembled a normal female distribution. Apparently, the variation in septal VP within the male gender seems to be as large as the variability in VP expression when males and females are compared.

The individual differentiation in lateral septal VP in relation to aggression in mice is based on the use of genetic selection lines. Although individual differentiation in aggressive behavior is a well-known phenomenon in a wide variety of animal species, one may wonder to what extent a similar relationship might exist in an unselected population. We studied this question in a strain of wild type rats (*Rattus norvegicus)* which is characterized by a strong variation in behavior and physiology between individual males. Fig. 1 shows the distribution in attack latency score in this population which was bred under standard conditions in the laboratory. The VP content of the lateral septum was measured by means of radio immunoassay of septal micro punches. It was demonstrated that non-aggressive males had a significantly higher VP

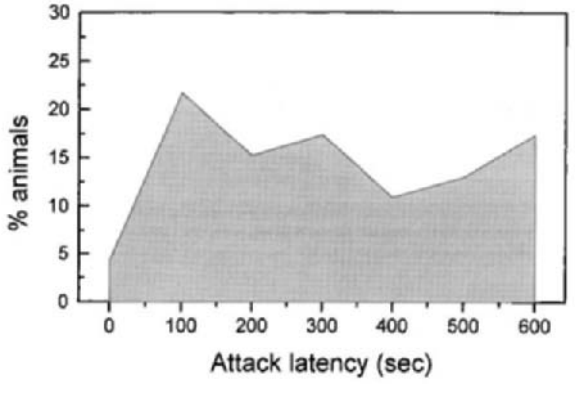

Fig. 1. Distribution of attack latency scores in a population of adult, laboratory bred wild type male rats.

content than aggressive males. This was confirmed by a quantitative immunocytochemical study (Everts and Koolhaas, 1997a). The non-aggressive males were found to have the highest density of VP immunoreactive fibers in the lateral septum. Fig. 2a shows a typical brain section at the level of the lateral septal area of the non-aggressive male, whereas Fig. 2b shows a brain section at the same level of an aggressive male. Fig. 2c shows the correlation of the quantified fiber density with the individual attack latency scores as measured several weeks before the immuno-cytochemical analysis. Clearly, highly aggressive males, characterized by a short attack latency show the least dense VP ir fibers. These results in male rats are similar to those observed in the selection lines of male mice, indicating that the relationship between aggression and LS VP may be more general.

One may wonder what the observed individual differentiation means for the total VP signaling in

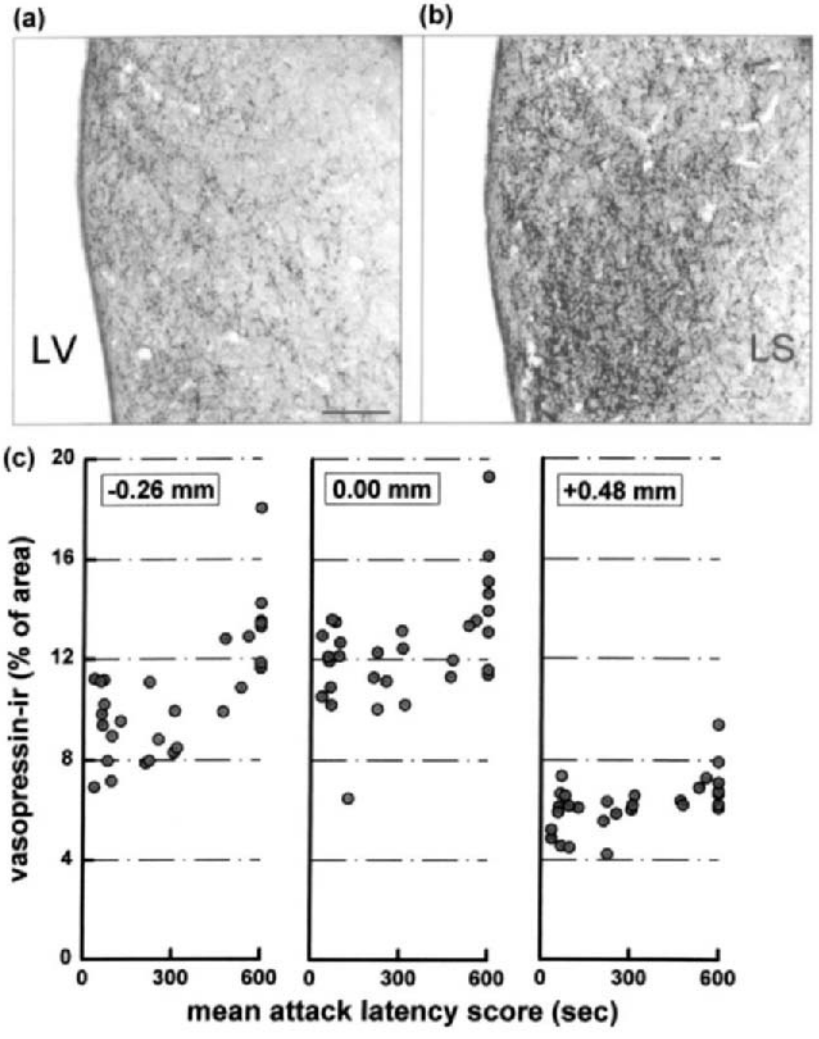

Fig. 2. Section of the lateral septum of an aggressive (a) and a non-aggressive (b) male rat showing the AVP-ir fibers. The quantified fiber densities at three different frontal planes of the brain are significantly correlated ($P < 0.01$) with attack latency (c).

the lateral septal area. Several possibilities arise: first, the differences may be due to differences in VP synthesis; second, it may reflect a difference in storage and/or release of VP; third, it may only reflect the size of the vasopressinergic network, while synthesis and storage/release are the same.

There are several arguments to suppose that this relationship is not due to a different degree of VP synthesis. First, synthesis differences are not very likely to be expressed in a differential fiber density. Second, there was no relationship with adult plasma testosterone (Everts and Koolhaas, 1997a). Therefore, it is more likely that the differentiation in lateral septal VP might have its origin in the neonatal period. Indeed, a study of the development of the genetically aggressive and non-aggressive male mice shows a differential prenatal and neonatal testosterone surge, suggesting that the processes involved in the sexual differentiation of the brain may be involved in the brain differentiation within the male gender as well (Compaan et al., 1993b). Studies are in progress to demonstrate that this differential course of perinatal testosterone is causally involved in the differentiation in aggressive behavior and lateral septal vasopressin.

The observed differences may be due to a differential rate of release and/or storage of the peptide in the LS. Optical density measurements obtained from mice (Compaan et al., 1993a) showed that more VP is present in fibers of non-aggressive animals compared to aggressive males. Moreover, studies on the role of T on VP expression indicate that the VP-ir in the lateral septal fibers reflects storage levels. Several castration and T replacement studies support this view (De Vries et al., 1985; Szot and Dorsa, 1994; Magnusson and Meyerson, 1996). Newly synthesized peptide may remain stored within the cell and its axons and terminals for prolonged periods of time. Taken together, this suggests that aggressive wild-type rats exhibit relatively low levels of lateral septal VP storage. This may also suggest that these animals show higher levels of release when compared with their non-aggressive counterparts. This is supported by a study of Landgraf et al. (1988), which shows that low contents of VP in distinct brain areas may well be linked to stimulated release patterns. In contrast to this line of argumentation, one would expect that VP released from a widespread and abundant fiber network (non-aggressive animals) would result in a larger effect than VP released from only a limited number of fibers (aggressive animals). Hence, on basis of these arguments and the present data it seems impossible to draw a conclusion on the existence of a differential VP signaling in the lateral septal area. Future VP mRNA studies should supply additional knowledge.

Although the significance of the individual differences in LS VP in male rats and mice for the total VP signal remains obscure, it remains a question to what extent the behavioral differentiation and lateral septal VP are causally related.

Functional aspects of lateral septal vasopressin

Several behavioral functions are shown to be mediated by lateral septal VP and some of these functions have been shown to be sex- and species-dependent.

There is experimental evidence for a function of VP in aggression and sexual behavior. The MeA/BNST occupies a central position in the circuitry that organizes sexual behavior and intraspecific aggression in the male rat and amygdalar VP was shown to play a role in these processes (Koolhaas et al., 1990; Minerbo et al., 1994). Microinfusion of VP into the LS resulted in an increase in aggressive behavior, but male sexual behavior was not affected (Koolhaas et al., 1991). A role in aggression is further supported by findings that VP is able to increase aggression-related flank marking in golden hamsters when applied into the LS (Ferris and Delville, 1994; Ferris et al., 1993). In another species, the prairie vole, lateral septal VP is strongly related to territorial aggression. It was demonstrated that VP antagonism blocked the transition to aggression after mating (Winslow et al., 1993; Wang et al., 1994a; Wang, 1995).

One of the best studied functions of lateral septal VP is its mediation of social memory as measured in the so-called social recognition paradigm (Thor and Holloway, 1982). This paradigm is based on the tendency of adult male rats to spend a great amount of time investigating young conspecifics. When exposed to the same juvenile for a second time shortly after a first exposure, but within 1 h, a

sharp drop in investigatory behavior will occur. This drop in behavior is absent when the adult is exposed to a different juvenile or when the inter-exposure time exceeds 1-2 h. This time-related social investigation of conspecifics is considered to be an ethological model for short-term social memory (Thor and Holloway, 1982; Gheusi et al., 1994) and it has been shown that VP is the key modulator of this behavior in males, but not in females (Bluthé and Dantzer, 1992; Landgraf, 1995; Engelmann et al., 1996). These studies revealed that peripheral, central, and local application of VP or related substances improves social memory. Application of VP receptor antagonists reduces the performance of adult rats to recognize previously met juveniles.

In line with the role of VP in social communication, but slightly different in character, is the involvement of septal VP in pair bonding and parental care as found in microtine rodents (Carter et al., 1995). In the monogamous prairie vole (*Microtus ochrogaster*), both males and females provide parental care. Male prairie voles that cohabited with a female for 3 days exhibited a higher level of parental behavior than sexually naive counterparts. This increased parental behavior coincides with increased septal VP release, as indicated by reduced septal VP-ir staining, increased VP mRNA expression in the BNST, and increased level of plasma T (Bamshad et al., 1994; Wang et al., 1994b). Injections of VP into the LS enhance parental behavior, whereas injections of the VP antagonist diminishes VP effects on parental behavior in male prairie voles (Winslow et al., 1993; Wang et al., 1994a). In meadow voles (*Microtus pennsylvanicus*), a promiscuous species that does not show any sign of pair bonding, males do not provide paternal care (male care towards litter). They show a lower density of VP-ir fibers in the LS as compared to male prairie vole. In addition, male meadow voles show no changes in their VP-ir staining in the LS or VP mRNA expression in the BNST after cohabiting with females or after becoming fathers (Bamshad et al., 1994; Wang et al., 1994a).

Recent studies suggest a function for septal VP in the mediation of emotionality, fear, and anxiety. It has now been shown that application of V_{1a} receptor antisense or antagonist into the LS results in a fear reducing (anxiolytic) effect. Surprisingly, application of synthetic VP fails to alter anxiety-related behavior (Landgraf et al., 1995; Miller et al., 1995; Liebsch et al., 1996). Earlier, lesion studies demonstrated the LS to be involved in the expression of fear and anxiety (Treit et al., 1993; Menard and Treit, 1996).

In a number of behaviors, the role of lateral septal VP is less clear or seems of less importance. Although VP has been implicated extensively in learning and memory processes in limbic systems, its effect in the septum is dependent on the task. Locally applied VP facilitates passive avoidance behavior (Bohus et al., 1993), and application of VP by a microdialysis probe did not impair acquisition of pole-jumping avoidance behavior. However, the application of a V_1 receptor antagonist impaired acquisition (Landgraf, 1995; Engelmann et al., 1996). That VP might mediate spatial learning originates from studies showing a detrimental effect LS lesioning as tested in the radial maze (Mharzi and Jarrard, 1992). Moreover, experiments have shown that VP administration into the septum impaired spatial learning, while VP antagonism left acquisition behavior undisturbed (Engelmann et al., 1992).

From this brief overview of the main functional aspects of VP in the LS in relation to behavior, it seems that a variety of functions can be distinguished. Lateral septal VP seems to modulate not only social behavior, but also fear and anxiety related behavior as well as spatial orientation. One may wonder whether these rather different behavioral effects of VP might have a common functional basis. To answer this question, one has to see how behavior in a variety of different social and non-social contexts may be interrelated.

Coping styles

Although animals are generally tested in one, well-defined behavioral paradigm, it is important to emphasize that the behavioral performance in one paradigm may predict the performance in another one. This is demonstrated in a number of studies which show that the individual level of aggressive behavior, i.e. the tendency to defend

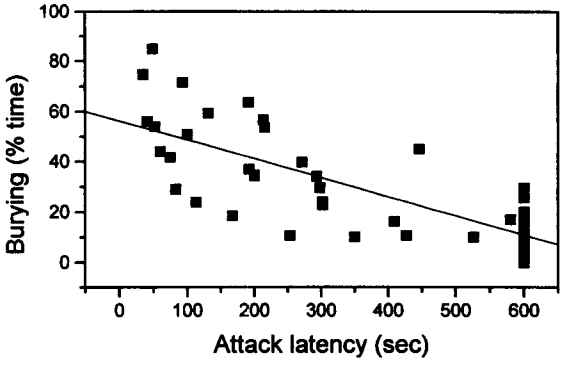

Fig. 3. Correlation between individual attack latency scores and burying behaviour in the shock-probe defensive burying task ($r = 0.73$, $P < 0.01$).

the home territory, is related to the way individual males react to environmental challenges in general.

For example, the large differentiation in aggressive behavior is correlated with burying behavior in the defensive burying paradigm. In this test, the animal is confronted with a small electrified prod in its home cage. Because this prod is a novel object, the experimental animal will explore it by sniffing at the object. Consequently, the animal receives a mild but aversive shock. As soon as it has experienced the shock, the animal has two options to avoid further shocks. It may either hide in a corner of the cage to avoid further contact with the shock prod, or actively bury the shock prod with the bedding material of the cage. Under these free choice conditions, aggressive males spend much more time burying than non-aggressive males (Fig. 3). Non-aggressive males remain immobile for quite some time. This demonstrates that the way an animal deals with its social environment (i.e. aggression) is related to the way in which an animal may deal with its environment in general. A review of the literature on selection lines of rats and mice suggests the same. Certain patterns of behavioral and physiological characteristics tend to be selected rather than a single, well-defined behavioral or physiological aspect. Apparently a co-selection for a wide variety of other aspects occurs at the same time.

This is further illustrated in Table 1, which summarizes the behavioral characterization of male mice and rats, when tested in a number of different situations.

Several conclusions can be drawn from Table 1. The correlations indicate that the individual level of aggressive behavior is apparently a reflection of the way in which the animals react more generally to a wide variety of environmental challenges. It seems that aggressive males have a more proactive type of behavioral response, in which they anticipate the situation and actively deal with the environmental challenge. The non-aggressive males on the other hand have a more reactive response and tend to accept the situation as it is. The data on cue dependency, obtained in several maze experiments show that aggressive males develop routines which means that behavior, once triggered, is very little affected by environmental stimuli. The behavior of non-aggressive males on the other hand is more guided by environmental stimuli. This latter aspect may be one of the most fundamental differences

Table 1
Summary of the behavioral differences between aggressive and non-aggressive male rats and mice

	Behavioral characteristics		
	Aggressive	Non-aggressive	References
Attack latency	Low	High	Oortmerssen and Bakker (1981)
Active avoidance	High	Low	Benus et al. (1989)
Defensive burying	High	Low	This chapter
Nestbuilding	High	Low	Sluyter et al. (1995)
Routine formation	High	Low	Benus et al. (1987)
Cue dependency	Low	High	Benus et al. (1987)
Conditioned immobility	Low	High	Bohus et al. (1987)

which seems to be important in the differential adaptive values of the two response types as well. These and other data support our view that the individual variation in aggressive behavior represents a variation in coping style which may be expressed in a wide variety of environmental challenges. It seems that this behavioral characterization is rather independent of the selection criterion used, suggesting that the characteristics of a coping style form a rather coherent set. When another behavioral characteristic is used as a selection criterion like nest-building or active shock avoidance, one seems to obtain two selection lines with almost the same characterization as obtained with the attack latency criterion (Benus et al., 1991; Sluyter et al., 1995).

In summary, the concept of coping style indicates that the same behavioral characteristic is maintained in a wide variety of contexts. In this view, aggressive behavior is instrumentally used by the organism to actively cope with a social challenge.

Lateral septal VP and coping style

One may wonder to what extent the correlation of the individual level of aggressive behavior and the size and distribution of the BNST–lateral septal vasopressinergic network as described earlier is functionally related to the differentiation in coping style. Since this question concerns the function of a single vasopressinergic subsystem, it can only be answered by local manipulation of the system.

In a series of experiments, we studied the role of lateral septal VP in a number of behavioral tests, using chronic local micro infusions by means of osmotic minipumps of a selective V_1 receptor antagonist ($d(CH_2)_5$-Tyr(Me) VP) in the lateral septal area of male rats. The animals were tested in a broad spectrum of behavioral tests including spatial learning tasks, social and object recognition, and several anxiety tests. Most of the experiments were performed to elucidate the functional role of lateral septal VP in aspects of coping behavior. Some of the main results will be presented here.

In view of the positive correlation between offensive aggressive behavior in the resident intruder test and defensive burying in the shock-prod burying test, one may expect to see effects of VP in this anxiety dependent test as well. However, local administration of the V_1 receptor antagonist did not affect shock-prod defensive burying and immobility behavior. Moreover, the chronic infusion of the V_1 receptor antagonist did not affect performance in a Morris water maze spatial orientation task. Hence, the treatment did not affect cue dependency, which is considered to be one of the major differences between the two coping styles. One may conclude from these negative results that vasopressin in the lateral septum does not have a general role in coping behavior.

In subsequent experiments, we tested the animals in a social recognition task. In accordance with the reports in the literature (Bluthé and Dantzer, 1992; Bluthé et al., 1993), the V_1 antagonist impaired social recognition. This effect appeared to be specific for social memory, because the recognition of objects was not affected when the animals were tested in a similar experimental setup as the social recognition test (Everts and Koolhaas, 1997b). When tested in the elevated plus maze, the local application of the V_1 antagonist has an anxiogenic action.

In sum, the data suggest that LS VP has a certain degree of specificity in social behavior and social learning tasks. The function in fear motivated behavior and spatial tasks remain unclear, due to contradictory results between experiments using the agonist or the antagonist. A selective role in social behavior seems to fit well to the specific neuroanatomy of the system. On the afferent side, the medial amygdala and the BNST receive direct input from the vomeronasal organ via the accessory olfactory bulb, a structure which is involved in the processing of species specific odors. Therefore, the individual differences in lateral septal VP may explain specifically only some of the social aspects of the differentiation in coping style.

Concluding remarks

Although in rats and mice the BNST lateral septal vasopressinergic network shows a strong individual differentiation in relation to aggressive behavior and coping style, the functional significance of this system in the organization of these behaviors is far from clear and may be limited to

the individual social capacities. Nevertheless, it is intriguing to see that the differentiation in vasopressin in relation to coping style seems to be more general extending beyond the lateral septal vasopressinergic complex. For example, in the selection lines of aggressive and non-aggressive mice, we observed higher numbers of vasopressinergic cells in the suprachiasmatic nucleus of non-aggressive mice (unpublished observations). This was also observed in mouse strains genetically selected for high and low nestbuilding, which is considered as again another aspect of coping (see Table 1) (Bult et al., 1992). In yet another model of coping style, i.e. rat strains genetically selected for high and low active avoidance, the number of vasopressinergic cells in the paraventricular nucleus was found to be highest in the low avoidance animals. These differences are thought to partially explain the differences in HPA axis reactivity observed in proactive and passive coping animals (Aubry et al., 1995). VP infusions into the central nucleus of the amygdala in these strains of rats appeared to affect heart rate and passive behavior in the low avoidance strains of rats in particular (Roozendaal et al., 1992) indicating a differential vasopressinergic modulation of this amygdaloid nucleus. These rather fragmentary observations suggest that the individual differentiation in vasopressinergic mechanisms may be more widespread throughout the central nervous system. In all these studies, it is the passive coping animal which expresses the highest number of VP cells or is most sensitive to the administration of VP. Unfortunately no systematic studies are available on the consistency of such a relationship in the various vasopressinergic subsystems in the brain. The observations nevertheless indicate that the size and distribution of the vasopressinergic system may be highly variable between individual males. This variation might explain some aspects of the differential capacity of individuals to cope behaviorally and physiologically with challenges in their social and non-social environment.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 5.5

Oxytocin and neuroadaptation to cocaine

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Oxytocin (OT) has been implicated in neuroadaptive processes such as learning, memory, and social-affiliative behavior as well as in the regulation of physiological responses leading to adaptation to the changing external and internal environment. Drugs of abuse constitute a major challenge to the homeostasis of the body and behavior. Drug tolerance, dependence and addiction may involve neuroadaptive mechanisms related to learning and memory at cellular and systems levels. Considerable effort has been made toward the understanding the neurobiological mechanisms of addictive behavior. Neuropeptides OT and vasopressin (VP) might be involved in these processes based on their effects on neuroadaptation and on their neuroanatomical localization and pharmacological actions. It has been demonstrated that both OT and VP have modulatory effects on opiate and alcohol tolerance and dependence. This chapter summarize the effects of OT, and in lesser extent VP, on neuroadaptation to cocaine, a psychostimulant drug of abuse. We have shown that OT inhibits acute cocaine-induced locomotor hyperactivity, exploratory activity and stereotyped behavior in rodents. Furthermore, OT facilitated, whereas VP inhibited the development of behavioral sensitization to cocaine. In a different model, OT inhibited the development of tolerance to the stereotyped behavior-inducing effects of cocaine as well as cocaine intravenous self-administration in rats. We demonstrated that OT acts through its specific receptors in the basal forebrain and in the hippocampus. OT and VP contents in the hypothalamus and limbic structures were altered by acute and chronic cocaine administration in a dose-dependent and region-selective manner. The differential plasticity of the brain OT-ergic and VP-ergic neurotransmissions in response to cocaine may underlie the differences in the involvement of these neuropeptides in cocaine addiction. Interaction of OT with dopaminergic neurotransmission in the nucleus accumbens, a key brain structure in drug addiction, as well as OT-ergic regulation of hippocampal processes may be among the mechanisms of action through which OT modulates neuroadaptation to cocaine. A better understanding of the role of OT in neuroadaptation to cocaine may provide an insight into both the mechanisms of neuropeptide actions in the brain as well as into the neurobiology of drug addiction.

Introduction

Since the discovery of the effects of neurohypophyseal peptides, arginine-vasopressin (VP) and oxytocin (OT), on learning and memory by De Wied and his coworkers, the effects and, in some cases, the physiological role of vasopressin-related peptides have been demonstrated in many different brain functions (De Wied et al., 1993). Besides their effects on learning and memory, vasopressin related peptides have been found to modulate sexual, social, reproductive, stereotyped and feeding behavior as well as fundamental physiological processes such as temperature and cardiovascular regulation and brain excitability. More recently, the effects of OT on rewarded behavior and opiate tolerance and dependence have been demonstrated (for review, see Sarnyai and Kovács, 1994) (see summary of results in Table 1). Briefly, subcutaneously (s.c.) administered OT dose-dependently attenuates or inhibits the development of acute (Kovács and Telegdy, 1987) and chronic (Kovács et al., 1985) tolerance to the analgesic effects of morphine and delayed the onset of the naloxone-precipitated withdrawal symptoms (Kovács et al., 1985). OT also inhibits the development of tolerance to the endogenous opiates β-endorphin (Kovács and

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Table 1
Summary of the effects of OT and VP on behavioral neuroadaptation induced by three major classes of drugs of abuse, opiates, psychostimulants and alcohol (see details in the text)

Drugs of abuse	Oxytocin	Vasopressin
Opiates		
Acute MO tolerance	– (Kovács and Telegdy, 1987)	0 (Kovács et al., 1987)
Chronic MO tolerance	– (Kovács et al., 1985)	+ (Krivoy et al., 1974; De Wied and Gispen, 1976)
MO withdrawal	– (Kovács et al., 1985)	?
β-Endorphin tolerance	– (Kovács, Telegdy, 1987)	?
Met-Enkephalin tolerance	– Schwarzberg et al., 1986	?
Cross-tolerance (μ/μ; μ/δ)	– (Krivan et al., 1992, 1995)	?
Heroin self-administration	– (Kovács et al., 1985)	– (Van Ree et al., 1988)
Psychostimulants (Cocaine)		
Locomotor hyperactivity	– (Kovács et al., 1990)	?
Exploratory hyperactivity	– (Sarnyai et al., 1990)	?
Stereotyped behaviour	– (Sarnyai et al., 1991)	0 (Sarnyai et al., 1991)
Sensitization	+(Sarnyai et al., 1992d)	–/+ (Sarnyai et al., 1991; Post et al., 1982)
Tolerance	– (Sarnyai et al., 1992d)	0 (Sarnyai et al., 1992d)
Self-administration	– (Sarnyai and Kovács, 1994)	– (Van Ree et al., 1988)
Alcohol		
Tolerance		
Hypothermia	– (Szabó et al., 1985)	– (Szabó et al., 1985)
Sedation	?	+ (Szabó et al., 1985)
Tolerance		
Development	– (Szabó et al., 1985)	– (Szabó et al., 1985)
Expression	– (Szabó et al., 1985)	+ (Szabó et al., 1985)
Maintenance	?	+ (Hoffman et al., 1978)
Self-administration	?	+ (Rigter and Crabbe, 1985)

Telegdy, 1987) and Met-enkephalin (Schwarzberg et al., 1986) as well as the cross-tolerance between two different opiates acting on the same receptor subtype (i.e. μ receptor) (Kriván et al., 1992) or on two different receptor subtypes (i.e., μ and δ receptors) (Kriván et al., 1995). The maintenance of intravenous (i.v.) heroin self-administration, but not the acquisition of this behavior, could also be inhibited by OT in rats (Kovács et al., 1985), which may indicate that OT probably acts on the tolerance/dependence processes rather than modifying the reinforcing properties of opiates per se. The effects of VP are much less studied in these paradigms. This neuropeptide stimulates acute morphine analgesia (Ratka and De Kloet, 1988) and facilitates the development of morphine tolerance (Krivoy et al., 1974). However, VP and its hormonally inactive fragment, des-glycineamide-arginine-vasopressin (DGAVP), inhibited heroin self-administration (Van Ree et al., 1988). The tolerance to the sedative effects of alcohol could be inhibited by OT and facilitated by VP, depending on the stage of its development (Szabó et al., 1986). On the other hand, VP facilitates alcohol self-administration in rats (Rigter and Crabbe, 1985).

However, effects of OT on rewarded behavior and drug tolerance/dependence may not be restricted to opiates or alcohol. This chapter summarizes results from our laboratory and from others that OT also interacts with behavioral changes related to cocaine addiction.

Cocaine addiction is a major health problem in the USA and other parts of the industrialized world

(Mendelson and Mello, 1996). Cocaine is a powerful psychostimulant with high abuse potential in humans (Mendelson and Mello, 1996). Its strong reinforcing effects and relatively short duration of action lead to repeated self-administration episodes, 'binges', and the very fast development of the addictive behavior. The acute effects of cocaine ('high') are characterized by strong initial euphoria with increased psychomotor excitation, decreased sense of fatigue, increased sexual drives and sometimes aggressive behavior in human users (Gawin, 1991). This initial phase is quickly followed by an acute withdrawal called 'crash', during which an intense anxiety develops (Gawin, 1991). To alleviate the 'crash' and to achieve the next episode of 'high', users administer further doses of cocaine, or they often take other drugs, such alcohol and opiates to balance the negative mood states (Gawin, 1991). Tolerance to the euphoria-producing effects of the drug appear relatively quickly which requires the administration of higher doses of cocaine to achieve the desired effects. As a function of chronic, high dose cocaine use, paranoid, 'schizophrenia-like' psychosis and epileptiform seizures may develop. These symptoms might be related to the sensitization phenomenon readily observed as increased psychomotor activity in rodent models of chronic cocaine administration (Post and Rose, 1976).

The primary neurochemical mechanism of action of cocaine is the blockade of the pre-synaptic dopamine transporter which leads to decreased reuptake and increased synaptic availability of the dopamine for the post-synaptic receptors (Kuhar et al., 1991). However, cocaine also blocks the reuptake of other monoamines, such as norepinephrine and serotonin. The role of these mechanisms of cocaine addiction is less well characterized (Galloway, 1988; Lakoski and Cunningham, 1988). The exact mechanisms of cocaine-related behavioral neuroadaptation, such as tolerance and sensitization have yet to be determined. It has been suggested that learning and memory processes may play an important role in cocaine addiction (Siegel, 1975, 1976; Baker and Tiffany, 1985). Since previous results suggested that OT has an inhibitory effect on memory processes and it has widespread effects on other neuroadaptive processes in the brain, probably through the modulation of the monoaminergic neurotransmission (Versteeg, 1983), we have hypothesized that OT might alter cocaine-related behavior (Fig. 1). The major goal of our studies was to study the role of OT in behavioral neuroadaptation related to cocaine in order to learn more about OT's effects on brain functions and the neurobiology of cocaine addiction.

Modification of acute and chronic behavioral effects of cocaine by oxytocin

The increased psychomotor activity is the most characteristic acute effect of cocaine in humans and in experimental animals (Koob and Bloom, 1988; Mendelson and Mello, 1996). Psychomotor stimulant effects of cocaine are in good correlation with the reinforcing properties of the drug. These effects share the same neural substrates and biochemical mechanism, namely the mesolimbic/mesocorticolimbic dopaminergic systems and the inhibition of presynaptic dopamine uptake, respectively (Koob

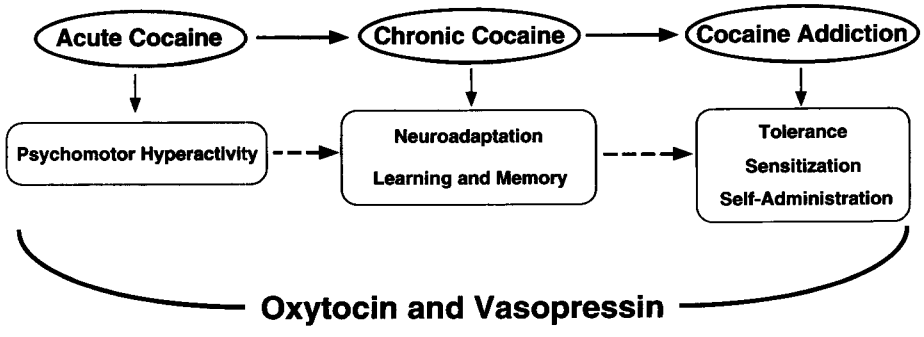

Fig. 1. Possible modulatory effects of oxytocin and vasopressin on cocaine addiction.

and Bloom, 1988). Therefore, psychomotor stimulant effects of cocaine have been widely used in behavioral models of cocaine's action in experimental animals. Low doses of cocaine (about 5 mg/kg; intraperitoneally, i.p.) facilitates locomotor activity in rodents, characterized by increased ambulation and horizontal activity. With the increase of the cocaine dose (5–10 mg/kg, i.p.), rearing episodes (vertical activity) and bouts of stereotyped behavior (grooming and sniffing) will appear. With further increasing the dose (15–30 mg/kg, i.p.), cocaine induces pronounced stereotyped behavior, dominated by intense sniffing and head bobbing in a restricted area of the cage. At the high end of the dose-response curve, cocaine doses higher than 40–50 mg/kg often produce seizure in rats. Several early data suggested that the two major forms of acute behavioral response to cocaine, the locomotor hyperactivity and the stereotyped behavior are mediated by separate neural systems. Neurochemical lesions of the mesolimbic DA terminal nucleus accumbens and nigrostriatal DA terminal caudate nucleus selectively block the locomotor stimulant and stereotyped behavior-inducing effects of cocaine, respectively (Kelly et al., 1975). Other studies, however, showed that these forebrain DA terminal structures may play a more complex role in the mediation of the behavioral effects of cocaine (Sarnyai, 1993). For example, sniffing behavior could be induced by local microinjection of cocaine into the mesolimbic nucleus accumbens, olfactory tubercle as well as into the nigrostriatal caudate nucleus, whereas grooming was elicited only after cocaine injection into the caudate nucleus in rats (Sarnyai, 1993). Similarly, electrolytic lesion of the nucleus accumbens inhibited sniffing behavior induced by apomorphine or amphetamine (Costall et al., 1975). Since cocaine-induced locomotor hyperactivity and stereotyped behavior are relatively well-characterized, these models have been chosen to study how OT interacts with acute behavioral effects of cocaine.

Cocaine produced a long-lasting (approximately 120 min) locomotor hyperactivity in a familiar environment (home cage) measured by an automated, microprocessor-controlled six-channel motimeter (Kovács et al., 1990). OT injections were administered in different doses (0.2–5.0 μg; s.c.) 60 min prior to cocaine. OT (1.0 and 5.0 μg) administration resulted in a dose-dependent decrease in cocaine-induced locomotor hyperactivity during the entire period of study (both the first and second hour). The lowest dose, 0.2 μg, did not alter cocaine-induced hyperactivity (Fig. 2, left). It has been shown that cocaine acts as a stressor leading to behavioral and neuroendocrine changes similar to that which occur after emotional stress (Moldow and Fischman, 1987; Rivier and Vale,

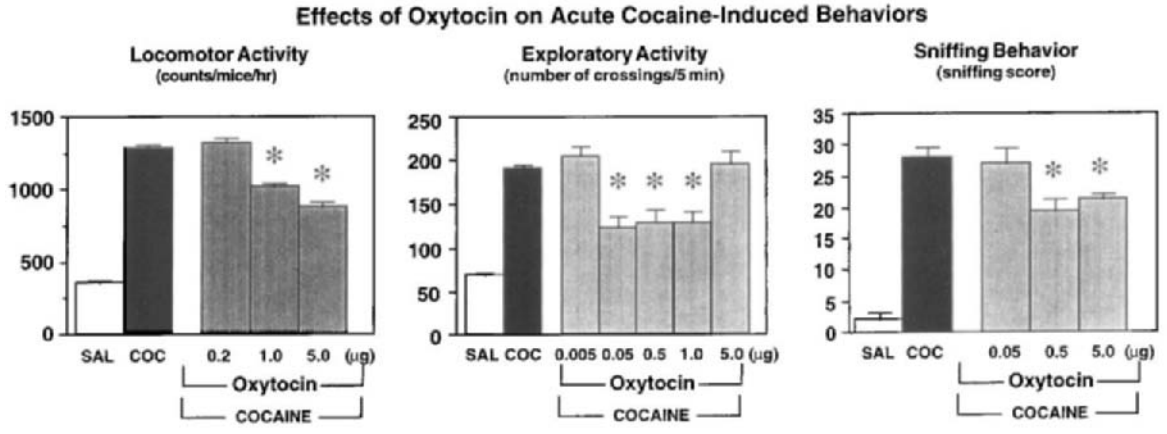

Fig. 2. Oxytocin inhibits acute behavioral effects of cocaine. (adapted from Kovács et al., 1990; Sarnyai et al., 1990, 1991). Abbreviations: SAL, 0.9% NaCl; COC, cocaine; *$P<0.05$ compared with cocaine-treated animals. See text for details.

1987; Sarnyai et al., 1992d). Moreover, cross-sensitization could be developed between the behavioral effects of stress and cocaine in rats (Deroche et al., 1995). Stress also facilitates acute behavioral effects of cocaine (Piazza et al., 1989). A novel environment is a powerful 'emotional' stressor for rodents. Mice showed a modest exploratory hyperactivity during the first 5 min in a non-familiar open-field (Sarnyai et al., 1990). In contrast, mice habituated to the open-field apparatus exhibited very low levels of exploratory activity in the open-field apparatus. Cocaine administration (7.5 mg/kg) 10 min prior to exposure to the novel open-field produced a 3–fold increase in crossing behavior, a measure of locomotor activity (Fig. 2, middle). Pretreatment with different doses of OT (0.05–1.0 μg) attenuated the cocaine-induced exploratory hyperactivity. However, OT did not alter exploratory hyperactivity induced by the novel environment itself (Sarnyai et al., 1990). Higher dose of cocaine (15 mg/kg) elicited long-lasting stereotyped sniffing behavior in rats (Sarnyai et al., 1991; Sarnyai, 1993). Different doses of OT (0.05, 0.5 and 5.0 μg; s.c.) were administered 60 min prior to cocaine injection. OT in 0.5 and 5.0 μg doses resulted in an attenuation of cocaine-induced sniffing behavior (Fig. 2, right panel). Structurally related neurohypophyseal peptides, arginine-8–vasopressin and lysine-8–vasopressin failed to alter cocaine-induced sniffing (Sarnyai, 1993). Grooming behavior can also be stimulated by lower doses of cocaine (3.75 mg/kg) in rats (Sarnyai, 1993). OT administration had no effect on this behavior, at least in doses that effectively attenuated cocaine-induced sniffing (Sarnyai et al., unpublished observation). It is interesting to note that OT altered locomotor hyperactivity and stereotyped sniffing behavior, which are mediated, at least in part, by the mesolimbic DA system, but it did not alter cocaine-induced grooming behavior, in which the nigrostriatal caudate nucleus serves as primary neural substrate.

Chronic cocaine administration may lead to a variety of different adaptive responses depending on the dose and routes of drug administration, treatment schedule or environmental factors. Tolerance is characterized by decreased responding upon chronic administration of a drug compared with the initial response, or by the requirement of higher doses of the same drug to induce responses equivalent to the initial response. During sensitization, an opposite adaptive phenomenon, repeated drug stimuli lead to augmented responding. Both sensitization and tolerance models were established to study the effects of neurohypohyseal hormones on neuroadaptation to chronic cocaine. Chronic, intermittent cocaine administration by repeated injections given in a non-familiar environment (7.5 mg/kg, s.c. twice a day for 5 days) produce sensitization in locomotor response when the effect of the challenge dose was tested 48 h after the last chronic cocaine injection in mice (Sarnyai et al., 1992b). Different doses of OT (0.005, 0.05 and 0.5 μg) were injected 60 min prior to each daily cocaine administration, except on the test day. OT administration did not interfere with the locomotor stimulatory effects of the test dose of cocaine. OT given in a dose of 0.5 μg facilitated the development of behavioral sensitization. In contrast, arginine-8–vasopressin, administered in a wide dose range (0.005, 0.05 and 0.5 μg), inhibited the development of sensitization to cocaine's locomotor stimulatory effects. The effects of pharmacological doses of vasopressin may be somewhat different compared with the effects of the endogenous peptide. It has been shown that behavioral sensitization to the locomotor stimulatory effects of cocaine could not be induced in Brattleboro rats lacking vasopressin synthesis, but this ability could be reinstated by administering vasopressin (Post et al., 1982).

Repeated administration of cocaine (7.5 mg/kg, s.c.; twice a day for 4 days) in a familiar environment (home cage) induced tolerance to the sniffing-inducing effects of cocaine in (Sarnyai et al., 1992a). This was indicated by a parallel right shift of the dose-response and time-effect curve of the test doses of cocaine (1.875, 3.25 and 7.5 mg/kg) injected on the fifth day of cocaine administration (Fig. 3A). The calculation of relative potency of cocaine has shown that a 6.42 times larger dose was required to produce the same effect in cocaine-tolerant rats as in cocaine-naive control animals. The development of tolerance was inhibited by pretreatment with OT (0.05 μg, s.c.) administered 60 min before each daily cocaine injection. OT

pretreatment shifted the dose-response curve for cocaine in cocaine-tolerant rats back to that of the control rats. The relative potency between the cocaine-tolerant and cocaine-tolerant plus OT-treated group was 5.3. This effect can be interpreted as an inhibition of the development of tolerance. OT alone had no effects on the sniffing-inducing effects of the single challenge dose of cocaine. A smaller dose of OT (0.005 μg) and arginine-8–vasopressin failed to alter the development of cocaine tolerance in this model.

Intravenous cocaine self-administration in rats is one of the most widely used animal models to study cocaine addiction. Rats quickly learn to self-administer cocaine into their jugular vein through an implanted cannula by pressing a lever in an operant chamber. The number of lever pressing for cocaine is a good index of the reinforcing efficacy of the drug. If an agent attenuates the reinforcing effects, the number of lever pressing for cocaine will decrease. Van Ree and his co-workers have found that the vasopressin neuropeptide desglycinamide$_9$,(Arg$_8$)-vasopressin (DGVP) decreased the acquisition of i.v. cocaine self administration in rats (de Vry et al., 1988). Furthermore, they also demonstrated that daily pretreatment with a vasopressin antiserum (i.c.v.) significantly increased self-administration (de Vry et al., 1988), which suggest a tonic inhibitory role of the endogenous vasopressin in cocaine self-administration. It was concluded that 'vasopressin neuropeptides may decrease the reinforcing efficacy of cocaine during the acquisition of self-administration rather than interact with nutritional and environmental factors influencing drug taking behavior' (van Ree et al., 1988). Based on these studies, we have conducted preliminary experiments to study the effects of OT on cocaine self-administration. After the stabilization of cocaine self-administration rate, OT pretreatment (0.5 μg, s.c.; 60 min prior to each self-administration session) resulted in a significant reduction (50%) of lever pressing for cocaine without altering the rate of non-reinforced lever pressing (Sarnyai and Kovács, 1994).

Taken together, these results indicate that OT has a general inhibitory effects on cocaine-induced behavioral responses, such as locomotor hyperactivity (Kovács et al., 1990), exploratory activity (Sarnyai et al., 1990) and stereotyped behavior (Sarnyai et al., 1991), and on adaptive response to repeated cocaine exposure, such as behavioral tolerance to the sniffing inducing effect of cocaine (Sarnyai et al., 1992a). OT may also attenuate the reinforcing efficacy of cocaine in a self-administration model (Sarnyai and Kovács, 1994). Interestingly, behavioral sensitization to the locomotor stimulatory effects of cocaine is facilitated by OT (Sarnyai et al., 1992c). In contrast, vasopressin does not alter behavioral effects of acute cocaine injection (Sarnyai et al., 1991) and cocaine tolerance (Sarnyai et al., 1992a) but inhibits the development of behavioral sensitization (Sarnyai et al., 1992c). Similar to OT, vasopressin neuropeptides also attenuate i.v. cocaine self-administration in rats (de Vry et al., 1988; van Ree et al., 1988).

Sites of action of oxytocin

Peripherally (s.c.) administered OT (in a microgram quantity) effectively modulated cocaine-induced behavioral changes (see above). One of the major problems of peripheral peptide administration is that larger peptide molecules can not easily penetrate the blood–brain barrier (Weindl, 1983). However, it was important to determine whether OT acts on peripheral target sites or does it pass the blood–brain barrier, at least in a small quantity, to bind to specific receptors in the central nervous system. To investigate this question, three different strategies were used. (1) Central (i.c.v.) pretreatment with a specific OT-receptor antagonist followed by peripheral OT administration. If the target sites of OT's actions are in the brain, the i.c.v. administered OT antagonist should inhibit the effects of peripheral OT treatment. (2) Intracerebroventricular (i.c.v.) administration of OT in nanogram quantities. If a very small dose of OT, which was ineffective when administered peripherally, is able to act on drug-induced processes after central (ICV) injection, this would argue for a central site of action of OT. (3) Local application of OT in a physiological dose range (picograms) into selected brain structures with and without high density of OT binding sites. If OT selectively modifies the drug-related behavioral processes when injected into a brain region which contains

high density of OT binding sites, this would suggest the existence of specific, intracerebral target sites for OT.

Central OT-receptor antagonist/peripheral OT

We hypothesized that a minor amount of SC injected peptide (or behaviorally active fragments thereof that can interact with cerebral OT binding sites) can pass the blood–brain barrier and reach brain target sites. To test this hypothesis, we designed a central OT-antagonist/peripheral OT strategy. The model of cocaine-induced sniffing behavior (Sarnyai et al., 1991) were used. *N*-α-(2–*O*-methyl-paratyrosine)-OT, an OT-receptor antagonist, was administered ICV at a dose of 50 pg, 15 min prior to SC OT injection. Peripheral OT administration was performed 60 min before acute cocaine injection. Cocaine-induced sniffing behavior was inhibited by peripheral pretreatment with OT in a dose of 0.5 μg. I.c.v. administration of the OT-receptor antagonist (50 pg) completely abolished the effects of peripherally injected OT (Fig. 4). This result could be explained by assuming a minor, but sufficient amount of OT (or its active fragments) administered peripherally can pass the blood–brain barrier and act on central nervous system target sites. The OT-receptor antagonist used in this study was effective on mammary gland myoepithelial tissue to block the effects of OT, in vitro (Krejci et al., 1973). The fact that the same antagonist was effective in the brain suggests that the central and peripheral OT binding sites could be homologous. The same OT-receptor antagonist injected into the nucleus accumbens potently blocked the effect of ICV administered OT on novelty induced grooming behavior (Drago et al., 1991). Caldwell et al., (1986) showed that a structurally different OT antagonist active on myoepithel cells inhibited the stimulatory effects of OT on novelty-induced grooming behavior when the antagonist was administered into the lateral ventricle. These data support the hypothesis that central and peripheral OT receptors may have structural and functional similarities.

Intracerebroventricular OT administration

If a small amount of OT is indeed able to cross the blood–brain barrier and bind to its receptors in brain sites, a fraction of the peripherally effective OT dose should be sufficient to alter cocaine-induced behavior when administered, by circumventing the blood–brain barrier, directly into the cerebral ventricules. Indeed, lateral ventricular microinjection of graded doses of OT (1–100 ng) resulted in a dose-related attenuation in cocaine-induced sniffing (Sarnyai et al., 1991). The effective i.c.v. doses (10 and 50 ng) were approximately one order of magnitude smaller than the effective peripheral doses (Fig. 4).

Local intracerebral OT administration

The effects of OT injected into different brain areas were studied in the models of cocaine-induced sniffing and chronic cocaine tolerance. The selection of the target brain areas was based on functional and autoradiographic localization of putative OT binding sites. To investigate the brain targets of OT's action on cocaine-induced stereotyped sniffing behavior, OT was microinjected into basal forebrain regions (nucleus accumbens, olfactory tubercle and posterior olfactory nucleus) and into the caudate nucleus, the regions which are involved in the mediation of sniffing behavior in rats (Sarnyai et al., 1991) (Fig. 4). OT (100 pg, 60 min prior to cocaine administration) microinjection into the nucleus accumbens resulted in a more than 50% decrease in cocaine-induced sniffing behavior. Cocaine-induced sniffing was also significantly attenuated by OT microinjection into the olfactory tubercle. The dose of OT used for the local microinjection was 10^2 times lower than the lowest effective dose injected ICV. Microinjection of OT into the olfactory nucleus, central amygdaloid nucleus, and caudate nucleus did not alter the cocaine-induced stereotyped behavior. The nucleus accumbens and the olfactory tubercle are the site of the termination of the mesolimbic dopaminergic projections (Lindvall and Björklund, 1978). The mesolimbic dopaminergic terminals in the nucleus accumbens are critically important in cocaine-related behavioral processes (Koob and Bloom,

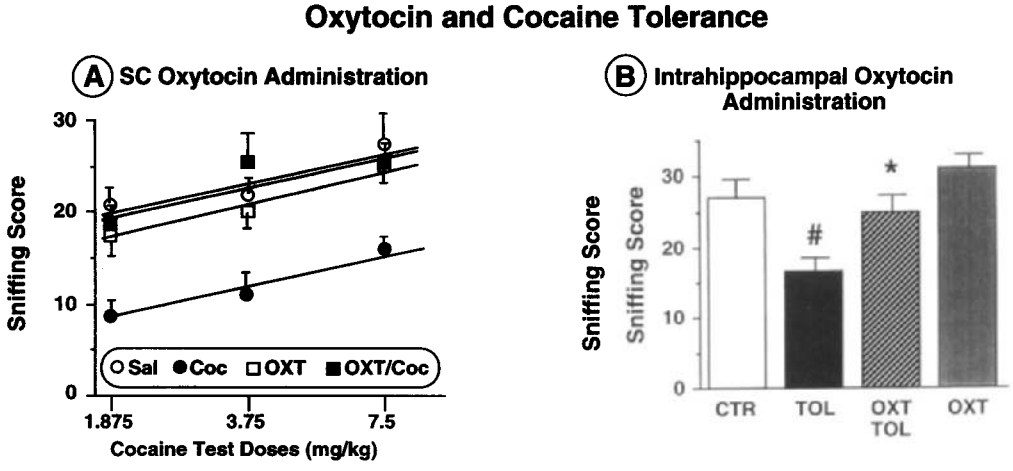

Fig. 3. Oxytocin attenuates the development of tolerance to the stereotyped behavior-inducing effects of cocaine. Adapted from Sarnyai et al. (1992d). (A) Effects of s.c. OT administration on cocaine tolerance. (B) Effects of intrahippocampal OT administration on cocaine tolerance. Abbreviations: Sal, 0.9% NaCl; Coc, cocaine; OT, oxytocin; #$P<0.05$ compared with controls; *$P < 0.05$ compared with cocaine-treated animals. See text for details.

1988). Dopamine-receptors in the olfactory tubercle are thought to responsible for the manifestation of sniffing behavior induced by dopaminergic drugs (Madras, 1984). These data strongly suggest the functional importance of OT receptors located in the basal forebrain areas in the mediation of the effect of OT on cocaine-induced stereotyped behavior.

Behavioral tolerance to chronic cocaine administration was inhibited by low doses of OT injected s.c. repeatedly before each cocaine injection (Fig. 3A). Furthermore, chronic cocaine administration resulted in a decrease in immunoreactive OT content in the hippocampus (Sarnyai et al., 1992d) (Fig. 5). To study the target site of administered OT and the role of hippocampal OT in the development of cocaine tolerance, OT was administered into the ventral hippocampus (Sarnyai et al., 1992a). The sniffing activity induced by the test dose of cocaine was significantly decreased at the end of chronic cocaine treatment, demonstrating the development of behavioral tolerance to cocaine (Fig. 3A,B). Intrahippocampal administration of 100 pg of OT 60 min before each tolerance-inducing cocaine injection almost completely prevented the development of cocaine tolerance, since the test dose of cocaine elicited the same sniffing activity compared with saline-treated, non-tolerant control (Fig. 3B). However, chronic intrahippocampal OT treatment did not alter the sniffing activity induced by the test dose of cocaine. These data showed that the ventral hippocampus plays a critical role in both the mediation of the effect of OT and the regulation of adaptive central nervous system processes related to behavioral tolerance to cocaine.

The brain regions selected as targets for OT microinjection are the parts of the terminal area of the mesolimbic dopaminergic system in the basal-forebrain (nucleus accumbens, olfactory tubercle, olfactory nucleus and amygdala), the major terminal region of the nigrostriatal dopaminergic system, such as the caudate nucleus, and a limbic structure, the hippocampus. Autoradiographic data showed a high density of OT binding sites outside the hypothalamus in among others, the nucleus accumbens, olfactory tubercle, central nucleus of amygdala, and the ventral hippocampus. OT binding sites were found in the caudate nucleus only in very low density (Brinton et al., 1984; De Kloet et al., 1985; Van Leeuwen et al., 1985; Freund-Mercier et al., 1987; Raggenbass et al., 1989). OT microinjection into the nucleus accumbens, the major terminal structure of the mesolimbic dopaminergic system containing OT binding

sites, inhibited cocaine-induced stereotyped behavior in rats (Sarnyai et al., 1991). Ventral hippocampus (subiculum) also contains a high density of functional OT receptors (De Kloet et al., 1985; Freund-Mercier et al., 1987; Raggenbass et al., 1989). Raggenbass et al. (1989) demonstrated that a positive correlation exists between neuronal responsiveness to OT and the presence of high affinity binding sites for this peptide in the ventral hippocampus by using light microscopic autoradiography and extracellular recording techniques. Intrahippocampal OT microinjection inhibited the development of behavioral tolerance to cocaine. OT microinjection into the brain regions without a significant number of OT-receptors (such as the caudate nucleus) failed to alter the central nervous system processes related to cocaine's effects.

Brain oxytocin content is altered by cocaine

If the role of endogenous OT is hypothesized in cocaine addiction, one might expect that acute and chronic cocaine administration should alter OT content in the hypothalamus and in behaviorally relevant extrahypothalamic brain regions. Changes in brain OT contents have been shown in response to environmental stimuli, such as stress (Miaskowski et al., 1988) and drug (opiate) administra-

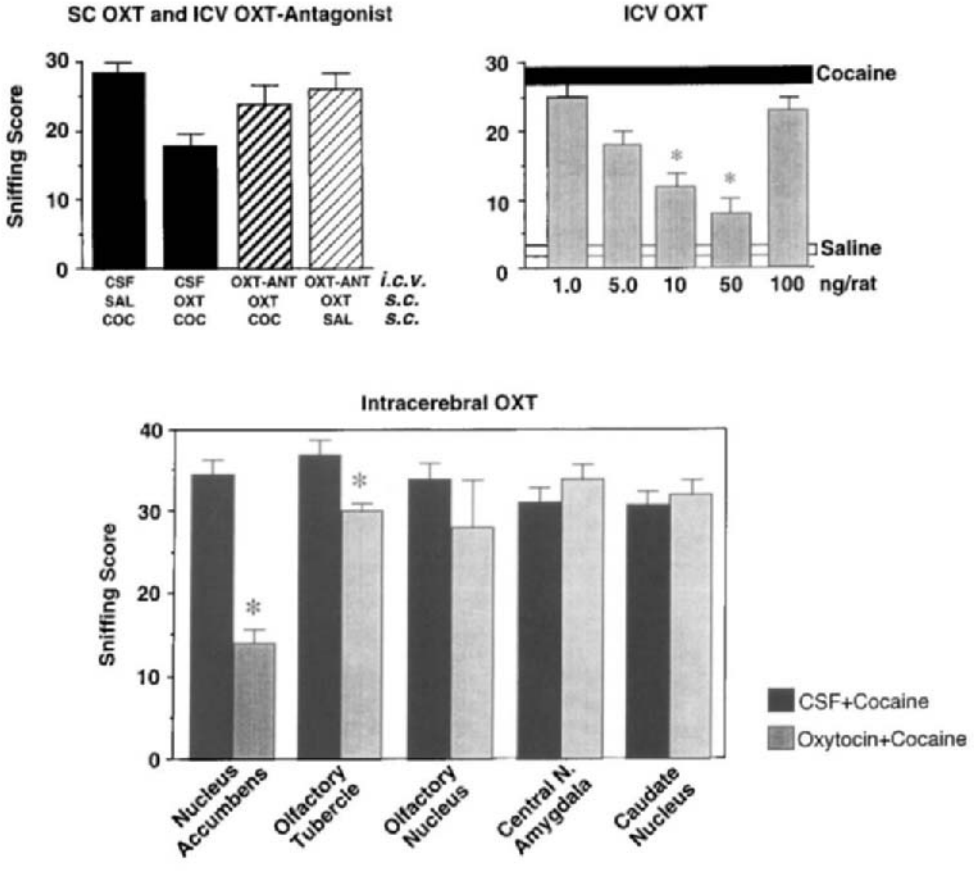

Fig. 4. Sites of action of oxytocin. Note that (1) an i.c.v. administered OT receptor antagonist attenuates the effects of s.c. injected OT, upper left; (2) OT administered i.c.v. inhibits the effects of cocaine in very low dose, upper right; (3) local microinjection of OT into the nucleus accumbens and olfactory tubercle inhibits cocaine-induced behavior, lower panel. Adapted from Sarnyai et al. (1991). Abbreviations: Sal, 0.9% NaCl; CSF, artificial cerebrospinal fluid; Coc, cocaine; OT, oxytocin; #$P < 0.05$ compared with controls; *$P < 0.05$ compared with cocaine-treated animals. See text for details.

tion (Sarnyai and Kovács, 1994). OT is thought to be involved in neuroadaptive changes to both stress and opiate administration. The effects of acute and chronic cocaine administration on plasma and brain OT contents were measured by radioimmunoassay (RIA). Acute administration of behaviorally effective doses of cocaine (7.5–30 mg/kg) increased the levels of OT in the hippocampus (Fig. 5 middle panel) in rats (Sarnyai et al., 1992c). OT levels were decreased in the basal forebrain regions (Fig. 5, right panel) in response to acute cocaine (Sarnyai et al., 1992c). Chronic cocaine administration (7.5 mg/kg, twice a day, for 4 days) resulted in a significant decrease in OT levels in the peripheral blood and in the hypothalamus, showing an OT depletion in the hypothalamo-neurohypophyseal system. OT was also depleted from the hippocampus by chronic cocaine administration (Fig. 5, middle panel), which can produce behavioral tolerance in rats (Sarnyai et al., 1992a).

Vasopressin (VP) levels in the plasma and vasopressin contents in different brain structures were also measured after acute and chronic cocaine administration (Sarnyai et al., 1992c). Acute, but not chronic, cocaine injection (7.5–30 mg/kg) increases plasma VP levels. In the brain, acute cocaine injection dose-dependently decreased VP content in the basal forebrain regions and in the amygdala, but not in the hypothalamus and in the hippocampus. Chronic cocaine administration resulted in a decrease in brain VP levels in the hypothalamus, basal forebrain, amygdala and in the hippocampus.

Others have also found alterations in plasma and brain OT in response to cocaine. Acute cocaine injection significantly elevated plasma OT concentration in baboons during late pregnancy (Morgan et al., 1996), which indicates that the effects of cocaine, described previously only in rats, could be generalized to primates and, possibly, to humans as well. Our data on the increased OT levels in the hypothalamus in response to acute cocaine was supported by the in vitro results of Sim and Morris (1992) who demonstrated that cocaine and dopamine increased Fos immunoreactivity, the product of an immediate-early gene *c-fos*, in OT cells from a neonatal hypothalamic paraventricular nucleus cell culture. Furthermore, in ovarectomized, estrogen-treated female rats, subchronic cocaine administration (15 mg/kg cocaine, s.c.; twice daily for 2 days) selectively decreased OT contents in the hippocampus but not in the amygdala and in the ventral tegmental area (Johns et al., 1993). The findings on changes in hippocampal OT contents corresponded well with our earlier results obtained by using a different cocaine treatment schedule and intact male rats (Sarnyai et al., 1992c).

The differential plasticity of the brain OT and VP systems in response to acute and chronic

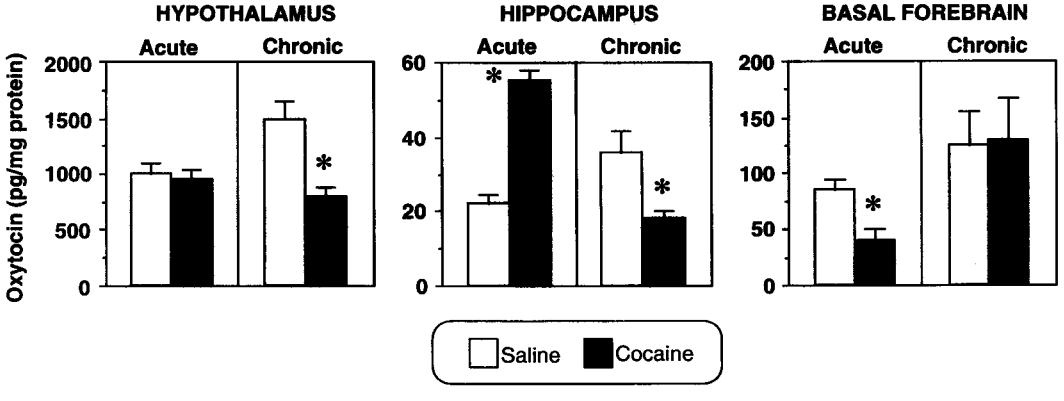

Fig. 5. Changes in brain OT contents by acute and chronic cocaine administration (7.5 mg/kg, s.c.). Adapted from Sarnyai et al. (1992c). Abbreviations: Saline, 0.9% NaCl; Cocaine, 7.5 mg/kg, s.c.; $*P < 0.05$ compared with saline-treated animals. See text for details.

cocaine administration may contribute in the behavioral neuroadaptation, such as the development of tolerance and sensitization and may explain the differential effects of OT and VP on these processes.

Mechanism of action: Role of dopamine

The dopaminergic system has been considered as a major brain target for cocaine. Cocaine acts on the presynaptic dopamine transporter protein to inhibit the reuptake of the transmitter, leading to increased availability of dopamine for the postsynaptic receptors (Galloway, 1988). Reinforcing and psychostimulant actions of cocaine are mediated mainly through the mesolimbic dopaminergic system (Kuhar et al., 1991). Two characteristic acute effects of cocaine, the locomotor hyperactivity and the stereotyped sniffing behavior were inhibited by OT administration (Kovács et al., 1990; Sarnyai et al., 1990, 1991). Locomotor activation by cocaine is mediated through the mesolimbic dopamine structures (nucleus accumbens), but probably both the nigrostriatal (caudate nucleus) and the mesolimbic (nucleus accumbens and olfactory tubercle) structures are responsible for cocaine-induced sniffing (Sarnyai, 1993). These results raised the possibility that OT may modify DA neurotransmission to alter neuroadaptation to cocaine. Behavioral pharmacological (Kovács et al., 1985; Sarnyai, 1993) and neurochemical (Kovács et al., 1990; Sarnyai et al., 1990) studies were conducted to elucidate the mechanism of action of OT. To behavioral pharmacologically target the pre- and post-synaptic dopamine receptors as well as dopamine release, the direct DA agonist apomorphine and the indirect DA agonist amphetamine were used, respectively. Low dose of apomorphine primarily stimulates pre-synaptic, inhibitory DA autoreceptors leading to decreased locomotor activity. In contrast, high dose apomorphine primarily stimulates post-synaptic DA receptors which result in increased locomotor activity. OT inhibited the hypomotility induced by a low dose of apomorphine but facilitated the high dose apomorphine-induced hypermotility in mice (Kovács et al., 1985). Neither stereotyped behavior nor locomotor hyperactivity induced by amphetamine were affected by OT administration in rats and mice (Kovács et al., 1985; Sarnyai, 1993). As it was shown, both cocaine-induced locomotor hyperactivity and stereotyped sniffing were inhibited by OT (Kovács et al., 1990; Sarnyai et al., 1990, 1991). These data suggest that OT administration does not act on DA release (e.g. no effect on amphetamine-induced behaviors) or on post-synaptic DA receptors, rather it modulates the pre-synaptic processes. Effects of OT on the behaviors induced by low and high doses of apomorphine should be interpreted as OT altered the DA re-uptake processes to inhibit the effect (or binding) of apomorphine. This may lead to the inhibition of apomorphine-induced hypomotility. Since the high dose of apomorphine acts on both pre- and post-synaptic DA receptors, and OT inhibits the inhibitory effect of the presynaptic autoreceptor stimulation, this could lead an increased net effect of apomorphine in OT-treated animals. Direct or indirect action of OT on cocaine-sensitive dopamine transporters on pre-synaptic DA terminals could not be ruled out.

Possible mechanisms of action of OT were further studied by using a false neurotransmitter, alpha-methyl-paratyrosine (α-MPT), which results in the enhanced disappearance of DA (increased DA utilization). Cocaine administration facilitated the α-MPT-induced dopamine disappearance in both the nucleus accumbens and the striatum (Kovács et al., 1990). SC administration of OT, in a dose which effectively antagonized the behavioral effects of cocaine, inhibited the effect of cocaine on dopamine utilization in nucleus accumbens but not in the striatum (Kovács et al., 1990; Sarnyai et al., 1990). Interestingly, although local cocaine microinjection (15 and 30 mg) into the nucleus accumbens, olfactory tubercle as well as into the caudate nucleus elicited the stereotyped sniffing behavior (Sarnyai, 1993), OT inhibited this effect of cocaine only when it was microinjected into the nucleus accumbens and olfactory tubercle, but not into the caudate (Sarnyai et al., 1991). One possible explanation of the lack of effect of OT on caudate nucleus could be the relatively low density of OT binding sites in this structure. The close correlation found between behavioral and neurochemical results suggest the role of mesolimbic dopamine

system in the mediation of the effects of OT on the acute behavioral actions of cocaine.

Chronic cocaine administration produced behavioral adaptation such as tolerance and sensitization, which could be inhibited or facilitated by OT, respectively (Sarnyai et al., 1992a,c). Although, the changes of dopamine neurotransmission in the nucleus accumbens as a possible neurochemical basis of neuroadaptation to chronic cocaine have been extensively studied (Johanson and Fischman, 1989), the exact mechanism of action of OT in these paradigms remains to be elucidated. However, results on the chronic inhibitory effects of OT on dopamine utilization, release and on postsynaptic dopamine receptors in the basal forebrain might explain the effects of OT on cocaine tolerance and sensitization (Kovács and Telegdy, 1983; Kovács et al., 1986). For example, chronic injection of OT (200 μg/kg; approximately 4 μg/mouse; SC) for 8 days decreased DA utilization in the basal forebrain, which contains the nucleus accumbens and the olfactory tubercle. Chronic in vivo OT treatment failed to influence in vitro uptake of [^{3}H]DA in basal forebrain slices. Spontaneous release of [^{3}H]DA (in the presence of 4.2 mM K^+) from the basal forebrain tissue was not affected by chronic in vivo OT treatment. The stimulated release of [^{3}H]dopamine (in the presence of 30 mM K^+) was significantly inhibited by chronic in vivo OT administration. Chronic OT treatment decreased the B_{max} value of [^{3}H]spiroperidol binding in the basal forebrain. However, the dissociation constant (K_d) of [^{3}H]spiroperidol binding was not influenced by OT. These data indicate that chronic, s.c. injection of OT is capable of influencing the DA neurotransmission in the mouse forebrain structures, which contain DA terminals of the mesolimbic DA-projection as well as oxytocinergic fibers and OT binding sites (De Kloet et al., 1985; Sofroniew, 1985; Freund-Mercier et al., 1987). Accordingly, chronic administration of the neuropeptide reduced the utilization and in vitro K^+-induced release of DA, as well as the density of [^{3}H]spiroperidol binding sites.

Dopaminergic neurotransmission is the major neural substrate for cocaine in the brain (Koob and Bloom, 1988). Alterations of gene expression and binding of dopamine transporter, pre- and postsynaptic DA receptors (D1, D2 and D3) in the mesolimbic dopaminergic system may contribute in the overall behavioral response to acute and chronic cocaine administration (Koob and Bloom, 1988; Kuhar et al., 1991). Our data suggest that OT selectively modulates DA neurotransmission in the mesolimbic structures, such as nucleus accumbens, which might explain the effects of OT on behavioral neuroadaptation to cocaine (Fig. 6).

Discussion: Putative neural pathways

Our present results along with others' suggest that OT plays a central role in organizing CNS responses to environmental challenges leading to behavioral adaptation or maladaptation, depending on the nature or the duration of the stimulus. Within this framework, the effects of OT on cocaine addiction may involve alterations in learning, neuroadaptation, memory or in reward related to cocaine. The key brain structures in the mediation of OT's effect on cocaine, i.e. the hippocampus, nucleus accumbens and olfactory tubercle, as well as the major neurochemical substrate of its action, the dopamine in the ventral striatal regions, seem to support these suggestions. However, neural pathways or a circuitry involved in OT's interaction with cocaine addiction have not been delineated. Here an attempt is presented to suggest a putative neural circuitry which may underlie such an interaction based on our own functional results and on recent morphological data on limbic-ventral striatal connections (Fig. 7).

OT containing fibers project to limbic brain regions, such as the hippocampus, especially to its ventral parts (Buijs, 1978; Sofroniew, 1985). OT receptors are located in the ventral hippocampus and microiontophoric application of OT excite non-pyramidal, inhibitory interneurons in this area (Raggenbass et al., 1989). Glutamatergic, excitatory neurons project to the nucleus accumbens-olfactory tubercle area (ventral striatum) from the ventral hippocampus (Kelley and Domesick, 1982). Activation of the excitatory input from the ventral subiculum by NMDA resulted in an increased neuronal activity in the nucleus accumbens and initiation of locomotor activity in rats (Wu and Brudzynski, 1995). Lesion of the ventral subiculum

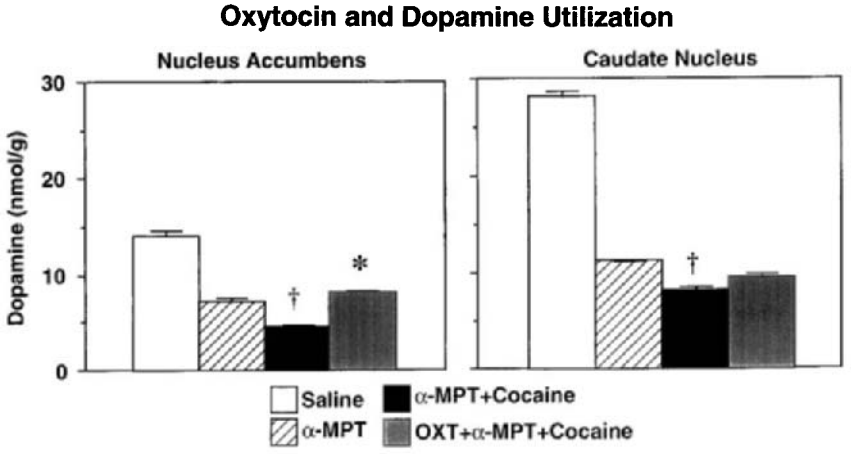

Fig. 6. Effects of oxytocin on dopamine utilization in the nucleus accumbens and in the caudate nucleus. Note that OT blocked the effect of cocaine on dopamine utilization only in the nucleus accumbens. Adapted from Kovács et al. (1990). Abbreviations: Saline, 0.9% NaCl; α-MPT, α-methyl-paratyrosine (250 mg/kg, i.p.) Cocaine, 30 mg/kg, s.c.; OT, oxytocin (1.0 mg/kg, s.c.); $\dagger P < 0.05$ compared with α-MPT-treated animals; $*P < 0.05$ compared with α-MPT plus cocaine-treated animals. See text for details.

completely abolished the locomotor response to intra-accumbens infusion of the psychostimulant amphetamine, in addition to blocking the potentiating effects of the same treatment on responding with conditioned reinforcement (Burns et al., 1993). Moreover, the amphetamine-induced increases in extracellular dopamine in the nucleus accumbens were blocked by ventral subiculum lesion (Burns et al., 1993). Furthermore, lesion of the hippocampus results in an increase in the rewarding or incentive motivational properties of a reinforcer (Schmelzeis and Mittleman, 1996). These morphological and behavioral results strongly suggest that the ventral hippocampus plays an important role in the modulation of ventral striatal/nucleus accumbens functions and in drug-induced psychomotor activation and reward. Nucleus accumbens and olfactory tubercle are particularly rich in OT receptors (De Kloet et al., 1985; Van Leeuwen et al., 1985; Freund-Mercier et al., 1987), making them a good target for both exogenously administered and endogenously released OT.

Neurons of the mesolimbic dopaminergic systems from the ventral tegmental area (VTA) project not only to the ventral striatum (Lindvall and Björklund, 1978), but also to the ventral hippocampus by forming the mesohippocampal dopaminergic projections (Gasbarri et al., 1997). Electrophysiological evidence suggests that dopaminergic neurons in the VTA could have a regulatory role in suppressing hippocampal excitability as demonstrated by decreased neuronal firing activity in the hippocampus after VTA stimulation or after local intrahippocampal DA administration (for review, see Gasbarri et al., 1997).

A simplified, putative neural pathway could be suggested as a possible morphological basis for the effects of OT on neuroadaptive processes related to cocaine (Fig. 7). From the hypothalamus, OT and VP pathways project to limbic structures, such as hippocampus and extended amygdala, which contain their receptors (Buijs, 1978; Sofroniew, 1985). OT excite GABA-ergic interneurons in the hippocampus, from where excitatory glutamate-ergic fibers project to the ventral striatum (Kelley and Domesick, 1982). These structures are functionally involved in the basic neurobiological processes of addiction (Koob and Bloom, 1988) such as motivation and reward (nucleus accumbens) and learning and memory (hippocampus and amygdala). Extended amygdala-ventral striatal structures receive innervation from the mesolimbic DA system, which is a primary target

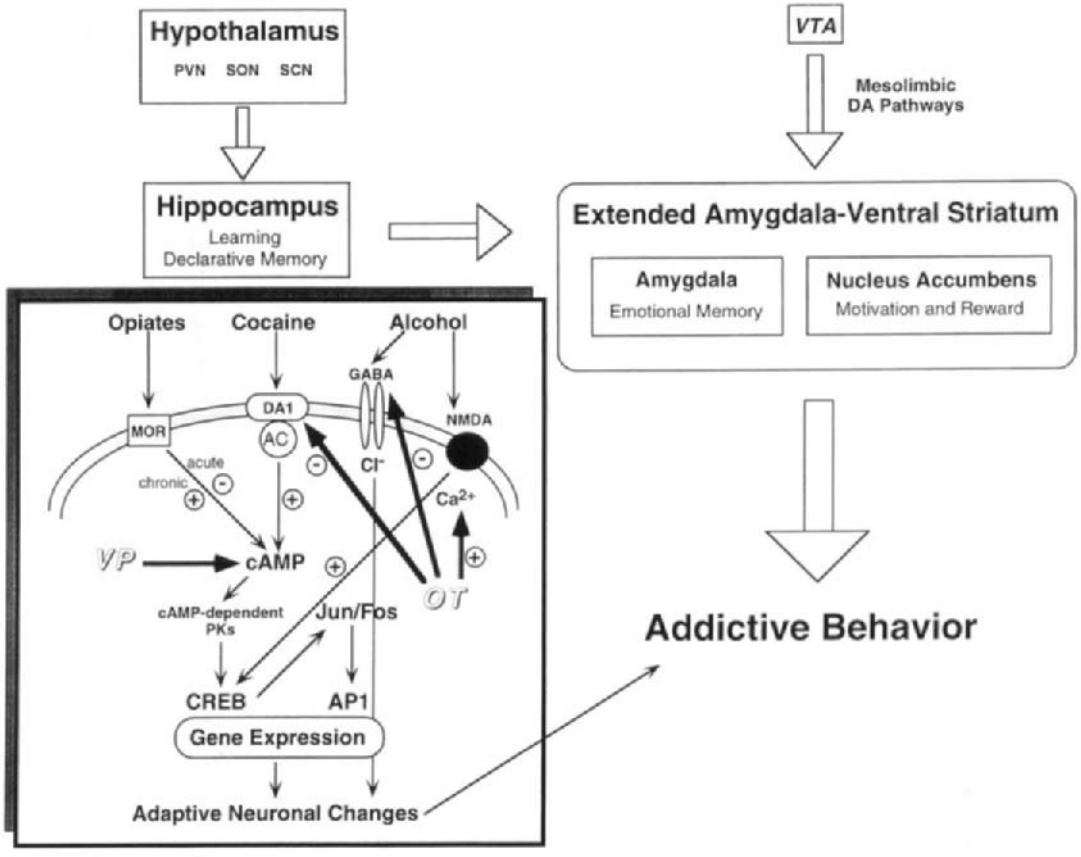

Fig. 7. Putative neural pathways and cellular mechanisms for OT's and VP's effects on neuroadaptation related to drugs of abuse. OT-ergic and VP-ergic projections from the hypothalamus terminate in the hippocampus, which is involved in learning and memory, as well as modulation of the activity of ventral striatal structures, such as amygdala and nucleus accumbens. These latter structures may be responsible for the emotional–motivational aspects of addiction and may be involved in the behavioral responses to reinforcing stimuli. The dopaminergic terminals in these ventral striatal regions from the VTA are the primary neural targets for cocaine's behavioral actions. At the cellular level (see inset) OT and VP may interact with numerous processes at the levels of receptors, second messengers or transcription factors to shape the intracellular adaptation to the drug stimulus. Repeated activation of these intracellular processes by drugs of abuse, such as cocaine, may lead to behavioral maladaptation resulting in the development of drug addiction (Nestler, 1997). OT may exert its effects on drug addiction through the modulation of a neural circuitry involving hypothalamus, hippocampus and extended amygdala-ventral striatal structures. Abbreviations: OT, oxytocin; DA, dopamine; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus; SCN, suprachiasmatic nucleus; VTA, ventral tegmental area; MOR, mu-opiate receptor; DA1, D1 dopamine receptor; AC, adenyl cyclase; PKs, protein kinases.

for drugs of abuse (Koob and Bloom, 1988). Cocaine, as an inhibitor of dopamine re-uptake, can modulate the functional activity of dopamine-sensitive neurons in the hippocampus and in the ventral striatum. During the course of chronic cocaine administration, adaptive changes in the DA-ergic neurotransmission may occur in these structures which may be related to behavioral changes such a tolerance and sensitization. OT and VP modulate DA neurotransmission in the ventral striatal structures (Versteeg, 1983). Therefore, it is hypothesized that these neuropeptides may act on neuroadaptive processes leading to drug addiction through limbic-ventral striatal

neural mechanisms. OT is released in response to acute and/or repeated cocaine stimuli from nerve terminals in the ventral hippocampus and in the nucleus accumbens/ventral striatum and then binds to its receptors in these nuclei (Sarnyai et al., 1992d). Similarly, exogenously administered OT could exert its effects through the same receptors in the limbic-basal forebrain structures. DA-ergic neurochemical events, such as release, receptor binding or metabolism in the nucleus accumbens and in the ventral hippocampus could be major targets for OT. It seems, that acute behavioral changes induced by cocaine are more sensitive to local OT administration into motor output structures, such as nucleus accumbens and olfactory tubercle (Sarnyai et al., 1991). Whereas neuroadaptation to repeated cocaine administration, such as behavioral tolerance, could be inhibited by OT injections into the hippocampus, which is thought to be involved in assessing changes in the environment, adaptation and cognitive functions (Eichenbaum et al., 1992). It is reasonable to assume that OT may act directly on the mesolimbic DA terminal structures to modulate the acute effects of cocaine, while during the development of cocaine tolerance, OT treatment may alter the regulatory effects of hippocampus on DA mechanisms in the nucleus accumbens (Drago et al., 1986; Kovács et al., 1990; Sarnyai et al., 1990). At the molecular level, adaptive neuronal changes in addictive behavior may involve alterations at the level of receptors (i.e. DA, GABA and NMDA), second messengers (i.e. cAMP), protein phosphorylation, release of Ca^{2+} and expression of immediate-early and late genes (Brinton and McEwen, 1989; Argiolas et al., 1990; Lambert et al., 1994; Brussard, 1995). However, the exact molecular mechanisms of OT's and VP's action on neuroadaptation to addictive drugs have not been extensively studied. This might provide new clues in our understanding of cellular and molecular mechanisms of adaptations.

One can speculate that limbic and basal forebrain neurons bearing OT receptors might be integral elements of the neuronal machinery involved in adaptive central nervous system responses to addictive drugs. The endogenous neuropeptide OT, as a physiological counterbalance mechanism, may play an important role in maintaining the functional integrity of neuronal processes affected by cocaine through its own receptors and by modifying the efficacy of a 'classical' neurotransmitter system, such as dopamine in the ventral striatum. However, the role of OT-ergic modulation of cocaine addiction in humans remains to be elucidated. With the advent of non-peptide analogs as possible pharmaceutical agents, OT or its active fragments may become an effective therapeutic tool to combat cocaine addiction and other addictive diseases.

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CHAPTER 5.6

A peptidergic basis for sexual behavior in mammals

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Vasopressin (VP) is a peptide neurotransmitter in the limbic system of rats. It is synthesized in the medial amygdaloid nucleus in the presence of sex steroids, transported to other limbic structures such as the hippocampus and septum and secreted there by a calcium-dependent process. In the hippocampus, VP acts on cerebral microvessels and local circuit interneurons. Its excitatory action on the inhibitory interneurons produces near-total shutdown of electrical activity of the efferent fibers of pyramidal cells, the projection neurons of the hippocampus. Stimulation of the medial amygdala and release of the endogenous VP duplicates these effects and, since they are blocked by ventricular application of a VP antagonist, the effects are almost certainly mediated by endogenous VP. Recording from the VP-containing cell bodies or of the hippocampal action of the peptide indicates that the system is selectively involved with the early stages of sexual behavior, specifically those appetitive behaviors that anticipate coitus. Stimulation of the VP cells produces alterations in sexual behavior in a manner consistent with the hypothesis that the medial amygdala organizes the appetitive phase of recognition of an appropriate partner and sexual arousal. This role for the medial amygdala complements the proposed role of nearby structures in the consummatory, reward and learned aspects of sexual behavior. Association between VP, oxytocin (OT) and homologs with sexual behavior is very widespread among vertebrates, including amphibians, reptiles, primates and humans. Humans and other primates display a phenomenon called 'concealed ovulation' that may have played a role in the evolution of social structures. The review concludes with a discussion of possible experimental strategies for evaluating the possible role of VP in concealed ovulation and other conditions in which sexual behavior occurs outside of estrus.

Introduction

Recent years have seen dramatic advancements in the understanding of the functions of brain vasopressin (VP) (reviewed in this volume) and in the understanding of the limbic organization of sexual behavior (reviewed in Everitt, 1990; Sachs and Meisel, 1988). The theme of the present review is that the two lines of investigation are convergent. In other words, workers examining the physiology of VP action in the brain have been led by the data to the unexpected conclusion that the circumstances of the peptide's release and the conditions of the peptide's action appear to indicate a particular role in sexual behavior. Similarly, some investigators of the brain structures and pathways subserving sexual behavior in mammals have come to believe that VP must mediate a critical connection in the system; specifically, a peptidergic link between the medial amygdaloid nucleus (AME) and other components of the limbic system.

We will organize our arguments for this thesis into five categories. In the first section we will present evidence that a peptide similar to VP is synthesized in neuronal cell bodies of the AME, that the synthesis is dependent on the presence of steroid hormones, and that the projection field of these neurons includes other limbic structures, such as the hippocampus. Then we will summarize the evidence taken from in vitro and in vivo experiments that a peptidergic signal within the limbic system is mediated by VP. In the ensuing sections

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we will cite results, taken from diverse sources, that relate VP and oxytocin (OT) to sexual behavior and that causally associate the AME and it's limbic influence with the generation of sexual behavior. In the latter case we will focus on the system that has yielded the best data, namely, the reproductive activity of the male rodent. Finally, we will attempt to place the foregoing research results on the limbic system into the context of other brain circuits responsible for sexual behavior, arguing that the VP-mediated link between the amygdala and the hippocampus may be responsible for those appetitive behaviors that are ultimately responsible for mating rather than those consummatory behaviors that are proximal to coitus. In concluding, we will describe potential extensions of these results to studies of primate sexual behavior.

Vasopressin in the medial amygdala and other limbic structures

In this section we will survey the evidence that the medial amygdaloid nucleus (AME) synthesizes a peptide similar to VP and sends it by axonal transport to the hippocampus. The sole source of hippocampal VP is the AME (Caffe et al., 1987). VP synthesis in the AME is sex steroid dependent and sexually dimorphic with males possessing more VP than females (Van Leeuwen et al., 1985). Both estrogen and testosterone receptors are present in the AME (Pfaff and Keiner, 1973; Sheridan, 1979). Estrogen receptors are expressed in vasopressinergic cells of the medial amygdala, suggesting a direct effect of the steroid upon these cells rather than a polysynaptic mechanism for the action on peptide expression (Axelson and Van Leeuwen, 1990). The promoter region of the VP gene contains two partial estrogen response elements, further supporting the suggestion of direct estrogen action (Szot and Dorsa, 1994). Upon entering a neuron in the AME, testosterone is most likely converted to estrogen or to dihydrotestosterone before interacting with the genomic response elements (McEwen, 1981).

Castration eliminates VP expression and treatment of gonadectomized male rats with estrogen in combination with dihydrotestosterone restores VP content to baseline level (De Vries et al., 1985, 1986; De Vries and Al-Shamma, 1990). When gonadectomized female rats receive estrogen in combination with dihydrotestosterone, the amount of VP mRNA expressed per cell in the AME does not differ from males, however these female rats have significantly fewer vasopressinergic cells in the AME than comparably treated male rats (Wang and De Vries, 1995). These results suggest that the number of vasopressinergic cells in an animal's AME is dependent upon testosterone exposure at an early developmental stage. In adult male rats the number of VP mRNA expressing cells in the AME correlates positively with plasma testosterone levels (Albeck et al., 1997).

Two potential mechanisms underlying gonadal steroid enhancement of VP transcription have been proposed. Both greater stability of VP mRNA (Carter and Murphy, 1991; Carrazana et al., 1988; Zingg et al., 1988), and increased transcription rate (Carter et al., 1993; Herman et al., 1991: Szot and Dorsa, 1994) have been reported. In contrast to their effects upon VP gene expression in the AME, gonadal steroids have little to no effect upon VP gene expression in the hypothalamus.

Glucocorticoids, often called 'stress' steroids, include corticosterone in the rat and cortisol in the human. VP transcription in hypothalamic nuclei is directly mediated by corticosterone (Albeck et al., 1994; Fig. 1). In the AME however, corticosterone does not directly affect VP expression but instead has a secondary effect. High plasma glucocorticoids inhibit testosterone synthesis (Monder et al., 1994), which in turn decreases VP transcription in the AME (Urban et al., 1991). Chronically stressed rats with high plasma corticosterone levels have few VP mRNA positive cells in the AME (Albeck et al., 1997) and exhibit deficits in sexual and aggressive behaviors (Blanchard et al., 1995). Sexual (Harris and Sachs, 1975; Albeck et al., 1991a: Baum and Everitt, 1992; Smock et al., 1992; Minerbo et al., 1994) and aggressive (Bolhuis et al., 1984; Luiten et al., 1985: Bluthe et al., 1990; Koolhaas et al., 1990) behaviors are thought to be mediated in part by vasopressinergic neurons in the AME.

Glucocorticoids may also affect vasopressinergic neurotransmission by mediating VP V_{1a} receptor expression. At physiological levels, glucocorticoids

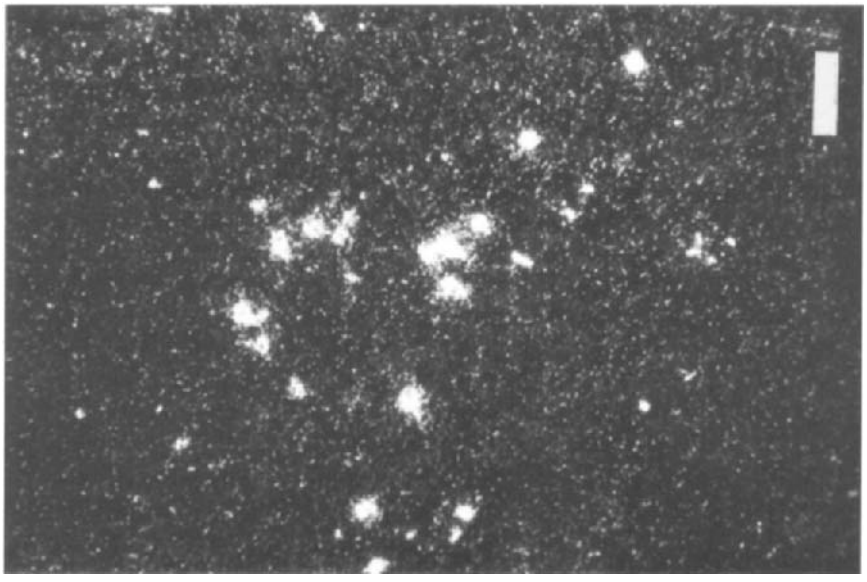

Fig. 1. VP mRNA in the medial amygdala detected by in situ hybridization. Rats were sacrificed by decapitation. Brains were quickly removed, frozen on powdered dry ice and stored at −70°C. The in situ hybridization technique used has previously been described in detail (Albeck et al., 1994). Sixteen micrometer coronal sections were cut on a cryostat and thaw-mounted onto gelatin coated slides. Sections were kept at −70°C until use. Slides were brought to room temperature in a dissector, fixed in 4% formaldehyde solution, rinsed in phosphate-buffered saline, acetylated, dehydrated in a series of ethanol baths, delipidized in chloroform, and dried. In situ hybridization for VP was performed using a 27-mer oligonucleotide probe directed against amino acids 110–118 of rat prepropressophysin. The 3′ end was labeled with [^{35}S]dATP using terminal transferase (Boehringer Mannheim) per manufacturers' instructions. Labeled probe was purified over a Nuctrap™ column (Stratagene). Typical activity was 3-5 × 10^5 cpm/microliter. Sections were hybridized in a solution of 50% formamide and 50% hybridization buffer [600 mM NaCl, 80 mM Tris–HCl, 4 μM EDTA, 0.1% sodium pyrophosphate, 0.2% SDS, 0.2mg/ml heparin and 10% dextran sulfate] at 42°C overnight. Subsequently, coverslips were floated off in 1× SSC and the tissue was washed four times for 15 min each at 45°C in baths containing 50% formamide and 50% 2× SSC. Slides were then rinsed in 1× SSC for 1 h at room temperature, dipped in ddH2O, dipped in 70% EtOH and dried. Slides were then dipped in Kodak NTB2 emulsion and exposed for 50 days. Emulsions were developed and counterstained with cresyl violet to histologically verify anatomical location. Darkfield photograph of VP mRNA expressing cells in the medial amygdala of the male rat. VP mRNA expressing cells are found throughout the medial amygdala, with a slightly higher frequency in the dorsal region. Scale bar, 50 microns.

increase the number of VP V_{1a} receptors expressed in a smooth muscle cell line without altering the affinity of these receptors (Colson et al., 1992), probably by facilitating transcription through increased mRNA stability (Murasawa et al., 1995). Glucocorticoids act in a dose-dependent manner since adrenalectomy (Saito et al., 1994) and supra-physiological levels of corticosterone both reduce the number of VP binding sites in rat hippocampus (Patchev and Almeida, 1995).

In rat hippocampus the V_{1a} 'vasopressor' receptor subtype is expressed by vascular cells (Kretzchmar et al., 1986) and induces microvessel constriction (Smock et al., 1987a; Cach et al., 1989). The V_{1a} receptor may be one of several VP receptor subtypes in the rat hippocampus. Based upon receptor binding data, VP in receptor heterogeneity within the hippocampus has been suggested (Poulin et al., 1988), consistent with reports that endogenous VP elicits an inhibition of CA_1 pyramidal cell field potential recordings (Albeck et al., 1990; Smock et al., 1990b), but has no effect upon dentate field potentials (Smock et al., 1991b).

Using the specific V_{1a} antagonist as a ligand

reveals a greater density of receptor binding in the dentate gyrus of the hippocampus than in area CA_1 (Phillips et al., 1988), and V_{1a} mRNA levels are higher in dentate gyrus than in CA_1 (Ostrowski et al., 1992; Szot et al., 1994). This same specific V_{1a} antagonist fails to block the endogenous VP-induced inhibition recorded in the CA_1 pyramidal cell area. However, this inhibition is blocked by a VP antagonist which has a higher affinity for OT receptors (Albeck et al., 1990; Smock et al., 1990b). Agonist and antagonist studies on the hippocampal receptor acted upon by VP, indicate that it differs from the V_{1a} receptor, having more OT receptor characteristics (Mühlethaler et al., 1983). The full extent of glucocorticoid regulation of vasopressinergic neurotransmission in the hippocampus remains to be investigated.

Vasopressin action in the limbic system

The bulk of what is known about neuropeptide physiology in the central nervous system really comes from pharmacology or neurochemistry. Candidate peptide transmitters are applied, in methods of greater or lesser technical sophistication, and neural or behavioral responses are measured. Binding studies and receptor autoradiography provide a biochemical complement to the understanding of post-synaptic processes in peptidergic transmission.

However, the independent manipulation of both pre-synaptic and post-synaptic cells, historically so crucial to the understanding of other transmitters such as acetylcholine, GABA and norepinephrine (Nicholls et al., 1992), have been conspicuously lacking in the study of central neuropeptides in vertebrates, though they have been conducted for some simple systems (Branton et al., 1978; Jan et al., 1979). Such studies accept as premises the pharmacology and neurochemistry of the putative neurotransmitter system, but go beyond these data to investigate the endogenously released substance, both by examination of the electrical activity of the pre-synaptic cells and by stimulation of the presumed peptidergic neurons. A critical feature of this truly physiological analysis is the use of a specific structural antagonist for the post-synaptic receptors, and the lack of such studies in the brain is probably due to a dearth of such antagonists for most neuropeptides.

Researchers in the field of VP and OT action are blessed with a number of such effective and specific antagonists and use of them has produced a rapidly growing and remarkably consistent body of literature describing the physiology of VP-mediated transmission in the limbic system. The purpose of the present section is to review these recent developments in specific connection to the peptidergic projection from the AME to the hippocampus in rats and to cite the specific experimental results that establish VP as the likely transmitter in the system. Given the cellular and molecular evidence for VP synthesis in the AME described in the last section, these results are best summarized as follows:

(1) Immunoreactive VP is released into the brain in a calcium-dependent manner indicative of neurosecretion (Buijs and Van Heerikhuize, 1978).

(2) Use of the rat hippocampal slice preparation as an accessible model for the peptidergic targets for VP reveals that the peptide, when bath-applied in known concentrations, has two primary effects. The first is an excitatory effect on tonically active interneurons, which yields an indirect inhibition of pyramidal projection neurons (Mühlethaler et al., 1982; Albeck and Smock, 1988; Smock et al., 1990a). The second is a constricting action on hippocampal microvessels (Cach et al., 1987a,b, 1989; Smock et al., 1987a,b; Topple et al., 1987).

(3) VP has the same neural action on the hippocampus in vivo when applied to the ventricles as it has in the slice in vitro (Albeck et al., 1990).

(4) Electrical stimulation of the VP-containing cell bodies in the AME produces an identical effect on the hippocampus to that obtained with applied VP (Albeck et al., 1990). Such an effect can only be obtained with electrode positions in the ventral AME, with nearby sites are ineffective (Albeck et al., 1990; Smock et al., 1991a). Fig. 2A shows the hippocampal effect of AME stimulation in the acutely anaesthetized rat. Either applied VP or endogenously released peptide can virtually eliminate all output from one of the two major projections from the hippocampus. Fig. 2B shows the effect of AME stimulation on hippocampal output in the awake, freely-behaving animal. Thus, the

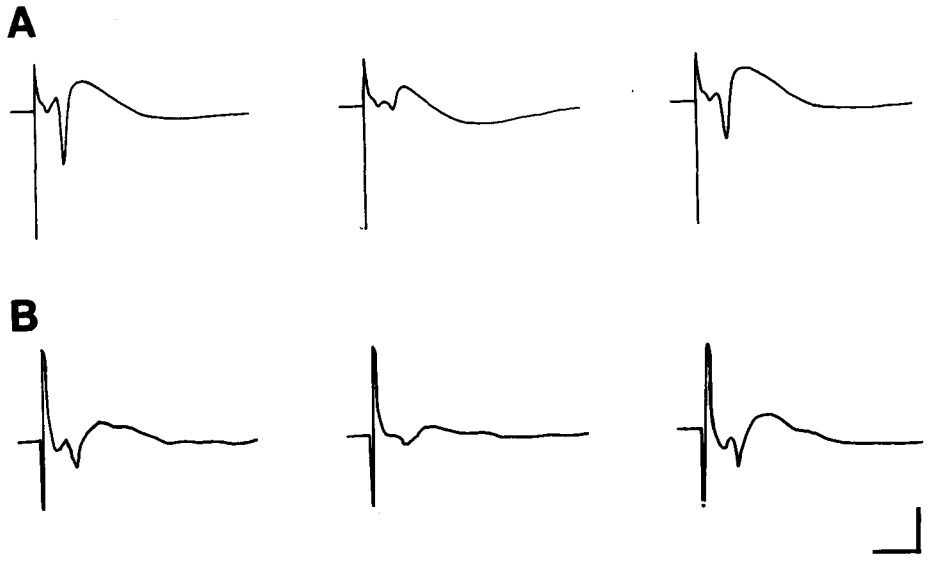

Fig. 2. VP-mediated inhibition in the hippocampus (A) anaesthetized male rat (B) freely behaving male rat. Figures are each (L to R) field potential averages before, during and after recovery from the affect of AME activation (5 V, 0.5 ms pulses, 100 Hz for 100 ms). Depression of downward deflection represents fewer action potentials leaving the hippocampus for each test pulse. (See Albeck et al., 1990; Smock et al., 1990b for details.) Scale bars, 4 mV, 10 ms.

same mechanisms may be at work in behavior just as in the anaesthetized, non-behaving animal and in the slice preparation (Albeck et al., 1991b).

(5) The limbic effect of AME stimulation resembles peptidergic transmission as it has been described in simple systems in three ways. First, single pulses of pre-synaptic stimuli are ineffective in eliciting a post-synaptic effect, which instead requires trains of stimuli to come about. This may reflect a tendency to conduction failure amongst the branches of small diameter, unmyelinated peptidergic fibers. Second, a progressive decrement in the amplitude of the hippocampal response is seen upon repeated presentation of the stimulus train. This may reflect depletion of a readily-releasable pool of peptide which can only be replenished upon de novo synthesis of precursor and axonal transport (Branton et al., 1978; Jan et al., 1979; Albeck et al., 1990). Third, the effect arises with a long latency (about 15 s) and lasts a long time (about 20 min), resembling the 'late, slow' potentials seen in simple systems that have been shown to be peptide-mediated. This may reflect a tendency for peptide transmitters to diffuse to targets from a remote site of release, a fact apparent in the hippocampus also since the VP-containing fibers are concentrated in the ventral portion of the structure but the putative peptidergic responses are found throughout (Jan et al., 1979; Caffe et al., 1987; Herkenham, 1987; Albeck et al., 1990).

(6) The mechanism for the hippocampal effect obtained with stimulation of the AME is almost certainly the same as that obtained with VP in the hippocampal slice since it entails excitation of individual interneurons and inhibition of single pyramidal cells in unit recording. Furthermore the inhibition of hippocampal output almost certainly reflects an increase in tonic inhibition of pyramidal cell somata, since other indices of hippocampal excitability are not altered by AME activation. These unaffected features include the stratum radiatum fiber volley, the dentate field potential, the pure post-synaptic potential and the latency and rate-of-rise of super-threshold synaptic potentials (Albeck et al., 1990; Smock et al., 1991a,b).

(7) In a combined immunocytochemical and single unit study, it was possible to antidromically activate the AME by stimulating the hippocampus,

eliminating the prospect of an unsuspected polysynaptic connection intervening between the structures. In this study the conduction velocity of the fibers was measured and was found to be in the range expected for small, unmyelinated peptidergic fibers. Immunocytochemistry of sections from electrolytically lesioned brains showed the single unit recording electrodes to lie amongst VP-containing cell bodies in the AME and stimulating electrodes to lie within a terminal field of small, unmyelinated VP immunoreactive fibers in the hippocampus (Albeck et al., 1993).

(8) There is no systemic change, such as in blood pressure, heart rate or body temperature, associated with the hippocampal response, effectively eliminating peripheral mechanisms, including those produced by circulating VP, as causes of the limbic effect (Raese et al., 1991).

(9) Just as for synthesis of VP, and VP mRNA, the hippocampal action presumed to be mediated by this peptide is wholly dependent on the presence of sex steroids. The signal is eliminated by castration in both genders, after an interval of about 13 weeks, and is restored upon hormone replacement (Albeck et al., 1991a: Smock et al., 1992). Fig. 3 shows the peptidergic action in an intact male (A) and a male 13 weeks post-castration (B).

(10) Though there is steroid-dependence in the system, there is no readily detectable difference in the strength of the signal between the genders, virtually complete shutdown of hippocampal output apparent in both male and female rats upon optimal AME stimulation (Albeck et al., 1991a). Moreover, as would be anticipated from the kinetics of the steroid-dependence, there is no significant alteration in the strength of the signal across the stages of the 4-day estrous cycle (Smock et al., 1992), nor are there any detectable alterations in the concentration of a variety of other candidate neurotransmitters or their metabolites across the estrous cycle (Desan et al., 1988).

(11) Most convincingly, the hippocampal responses that follow AME stimulation are almost certainly mediated by VP since they are invariably blocked by ventricular application of a structural antagonist for the peptide. This is true for AME effects on single units in the hippocampus (Albeck et al., 1993) and for hippocampal field potentials (Smock et al., 1990b; Albeck et al., 1991a) and can be demonstrated using internal controls (repeated stimuli in one animal) and external controls (comparison with an ineffective antagonist, Smock et al., 1990b). Blockade of the inhibition by the antagonist is complete, indicating that a co-transmitter is unlikely. However, residual excitation after blockade of the peptidergic inhibition may reflect action of a co-transmitter or, as has been argued, a reflection of the vascular action of the peptide (Smock et al., 1990b).

(12) Many of the salient results cited in this

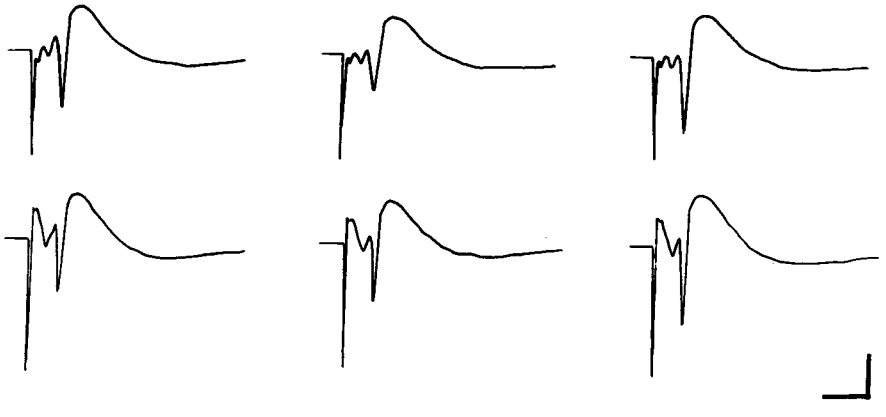

Fig. 3. P-mediated inhibition in the hippocampus in intact males (A) but not castrated males (B). As for Fig. 2, but AME stimulation is 13 V (A) and 15 V (B). (See Albeck et al., 1991a; Smock et al., 1992.) Scale bars, 4 mV, 10 ms.

section, including the action of the endogenous peptide on single units and field potentials as well as blockade of the limbic responses with a specific VP antagonist, are readily and routinely reproduced by undergraduates as laboratory exercises (see Goldberg et al., 1993, for discussion).

In sum, a large body of physiological results complement secure anatomical, neurochemical and pharmacological data to indicate that a peptide similar to VP mediates an important influence by the amygdala on the output of the hippocampus.

Brain vasopressin, brain oxytocin, and sexual behavior

The association between VP-like peptides and sexual behavior is very thorough-going among the vertebrates, suggesting that the origins of the peptide family as mediators of reproductive activity must be very ancient. Homologs of the mammalian peptides OT, arginine-VP and lysine-VP are found in birds, reptiles, amphibians, teleost fish, chondrichthyes and even jawless fish (Moore, 1992). Described by names such as 'vasotocin' and 'mesotocin' to indicate partial common sequence with the mammalian peptides, a particularly large amount of information has been gathered about the behavioral action of 'arginine vasotocin' (AVT) in amphibians and reptiles (Moore, 1992). AVT injections in male newts elicits clasping behaviors typical of mating, whereas AVT blockers eliminate such behaviors (Moore, 1992). In frogs, AVT elicits mating calls in males (Penna et al., 1992; Boyd, 1994) and appears to play a role in female mating behaviors as well (Diakow, 1978; Boyd and Moore, 1992).

Mating calls in anurans also play a role in territoriality and aggression, and studies of VP action on these sexually-related social behaviors have also been conducted in mammals. For example, in golden hamsters arginine VP elicits the territorial display of flank-marking, and a VP antagonist can disrupt dominance relationships in males (Ferris, 1992). In female mice brain OT has been proposed to mediate maternal behaviors (Pedersen et al., 1992). In monkeys, OT and VP are thought to influence social status (Winslow and Insel, 1991), and in monogamous animals, such as prairie voles, brain VP is thought to initiate pair-bond formation (Winslow et al., 1993; reviewed in Carter et al., 1995). These latter studies are especially intriguing since they suggest a role for the peptides in those aspects of social behavior, involving both attraction and aggression, that appear early in the sequence leading to conception and reproduction. In this manner they are consonant with findings regarding the circumstances of endogenous VP release in the brain (reviewed below).

Finally, there are ancillary data from a number of different sources that support a role for VP and/or OT in sexual behaviors. Plasma and CSF levels of these peptides have been reported to increase after sexual activity in a number of species (Sharma and Hays, 1977; Stoneham et al., 1985; Hughes et al., 1987) and after orgasm in people (Carmichael et al., 1987; Murphy et al., 1987). Most importantly for what is to follow, central injections of OT can elicit sexual behavior in male rats (Arletti et al., 1985; Melis et al., 1986) and the specific antagonist shown to completely block the peptidergic signal from the AME to the hippocampus (reviewed in the last section) has also been shown to attenuate sexual behavior in male rats (Argiolas et al., 1988).

The medial amygdala and sexual behavior

The amygdaloid complex, as part of the limbic system, is a collection of nuclei conventionally grouped together on the basis of neuroanatomical connectivity and cytoarchitecture. Historically, the amygdala has been partitioned into three general sections; the corticomedial, the basolateral, and the central divisions. These divisions reflect differences in both efferent and afferent connectivity. The basolateral nuclear group receives projections primarily from the neocortex, while the olfactory bulbs project to the corticomedial group and the central nuclei maintain a reciprocal connection with autonomic regulatory circuits in the brainstem (Noback and Demarest, 1977; De Olmos et al., 1985; McGregor and Herbert, 1992). This taxonomy maintains a functional as well as structural significance. The differences in input suggest that the separate amygdaloid nuclei play different roles in the neural regulation of incoming sensory information.

The amygdaloid complex has long been asso-

ciated with central processes mediating sexual behavior in mammals. Although damage to the amygdala and pyriform regions has been shown to result in behavioral hypersexuality in male primates and felines, this effect has not been reliably produced in rats (Kluver and Bucy, 1939; Schreiner and Kling, 1953, 1954; Masserman et al., 1985: Kling, 1968). Studies examining the effects of localized lesions of the various nuclei of the amygdala in sexually experienced male rats have shown various behavioral effects dependent upon the specific lesion site. There is evidence that indicates that lesions confined to the basolateral nuclei of the amygdala produce a decrease in inter-male aggression while leaving measures of sexual behavior virtually unaffected (Harris and Sachs, 1975; McGregor and Herbert, 1992), and other results with excitotoxic lesions that suggest the basolateral nuclei may mediate that portion of appetitive sexual behavior involved with conditioned incentives for gaining access to a female (Everitt et al., 1989; Everitt, 1990). Corticomedial lesions of the amygdala have resulted in a generalized disruption of the appetitive and, to a somewhat lesser extent, the consummatory phases of male sexual behavior while producing little or no change in terms of intermale aggression (Giantonio et al., 1970; Harris and Sachs, 1975; Kondo, 1992; McGregor and Herbert, 1992). A further separation in behavioral consequence appears to exist between lesions made to the cortical and the medial amygdaloid nuclei. It has been reported that lesions made specifically to the medial amygdaloid nucleus (AME) results in complete elimination of copulatory behavior in sexually inexperienced male rats, whereas cortical lesions produce significantly milder suppression of sexual behavior characterized by a reduction in mount frequency and failure to copulate to ejaculation (Kondo, 1992). This result suggests that there is a functional difference between the cortical and medial nuclei of the amygdala.

Both the cortical and the medial nuclei of the amygdala receive projections from the olfactory bulbs and, in turn, project to other limbic structures that have also been implicated in mediating sexual behavior such as the medial preoptic area of the hypothalamus (mPOA) and the hippocampus (Scalia and Winans, 1975; Kevetter and Winans, 1981a,b; De Olmos et al., 1985; De Vries et al., 1985: Caffe et al., 1987; Sachs and Meisel, 1988). Evidence suggests that the AME maintains a functional association with the mPOA in mediating neural events necessary for the normal expression of sexual behavior. In a study conducted by Kondo and Arai (1995), male rats received a unilateral lesion in the mPOA which produced a reduction of intromission behavior versus sham-operated controls. A unilateral AME lesion was then made ipsilateral or contralateral to the initial mPOA lesion. Contralateral lesions greatly reduced all aspects of sexual behavior while ipsilateral lesions did not further reduce sexual performance over the reduction induced by the initial mPOA lesion alone. Lesions to the mPOA have been shown to produce a reduction in the consummatory aspects of male rat sexual behavior while leaving measures of sexual motivation and appetitive behavior essentially intact (Larsson and Heimer, 1964; Lisk, 1968; Giantonio et al., 1970; Christensen et al., 1977;). Stimulation of the mPOA has been reported to accelerate ejaculation and mount latency in male rats as well as reduce the post-ejaculatory interval (Malsbury, 1971). That lesions to the AME reduce the appetitive, as well as the consummatory, aspects of sexual behavior suggests that it has perhaps a different role than the mPOA in the central mediation of these events.

Significantly, the AME is among the limbic structures that react in immunocytochemistry for c-Fos, the transcription factor for the early intermediate gene *c-fos*, upon increases in sexual behavior. Such increases are thought to reflect increases in electrical activity and are seen in male rats after sexual activity (Baum and Everitt, 1992) and, for the AME of male gerbils, specifically around the period of ejaculation (Heeb and Yahr, 1996).

Given these anatomical and lesion data implicating the AME in sexual behavior, we commenced a series of studies on freely behaving male rats using recording and stimulation techniques to assess the function of the structure. The results of these experiments can be summarized as follows:

(1) Bilateral stimulation of the AME in isolated males causes a reorganization of behavior that resembles the post-ejaculatory interval as it has been described in the literature (Sachs and Meisel,

1988). Typical behaviors of sleeping, grooming and exploration are replaced by a 'freezing' behavior that is punctuated by bouts of rearing and sniffing. At the same time there is a hippocampal response of the kind we have shown to be VP-mediated (Albeck et al., 1991b; Fig. 2).

(2) The electrical stimulation appears to be rewarding in male rats since they will seek out the location in an open field or Y-maze where a previous stimulus was delivered (Albeck et al., 1991b).

(3) Two other features of the post-ejaculatory interval, ultrasonic vocalizations at 22 kHz and a slow-wave EEG, are also seen in the interval of reorganized behavior and hippocampal inhibition following AME stimulation (Smock et al., 1992).

(4) Lower levels of electrical stimuli produce more subtle and fragmented responses. For example, with low level AME activation appetitive sexual behavior is modulated in such manner that male rats engage in more sexual behavior with conspecifics that normally elicit low levels of

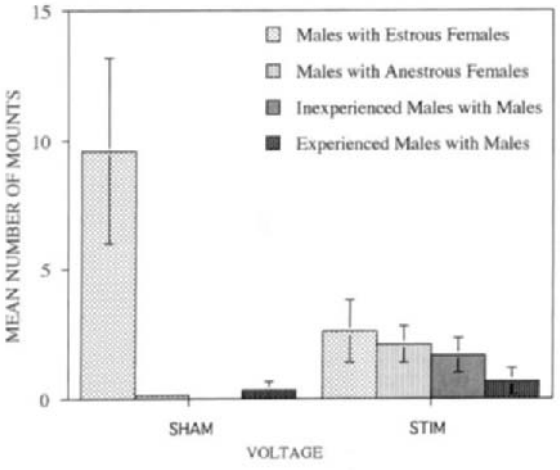

Fig. 4. Bilateral AME activation increases infrequent sexual behaviors and decreases frequent sexual behaviors. Mounting frequency is shown in male rats implanted bilaterally with bipolar concentric stimulating electrodes in the AME, for a period of 30 min. post-stimulation exposure to (L to R) primed females, unprimed females and other males (male subjects being experienced or inexperienced). Usually, mounting occurs with receptive females only, but upon AME activation (second set of columns) it occurs in the other conditions as well.

such contact (i.e. unreceptive females and other males). Conversely, low levels of AME activation cause males to exhibit less sexual attraction to conspecifics that normally elicit high levels of contact (i.e. receptive females). Some of these data are shown in Fig. 4 (Stark et al., 1998a,b).

(5) A chronic single-unit recording from the presynaptic structures in the AME shows that the peptidergic cells become active early in sexual behavior, shortly after presentation of a receptive (but not an unreceptive) female but well before mounting, intromission or ejaculation (Minerbo et al., 1994).

(6) Chronic field potential recording from postsynaptic structures in the hippocampus shows the presumed peptidergic effect to arise also during early phases of sexual behavior such as grooming and mounting. However, hippocampal inhibition in this study was also seen in other social, but nonsexual, behaviors such as fighting between males. This latter effect has a different waveform than the peptidergic one we have studied and may be mediated by a different transmitter (Garritano et al., 1996).

(7) The behavioral data is best interpreted if male rodent sexual behavior is considered as a reaction chain of fixed action patterns beginning with approach, investigation and mounting and ending with recumbency and the post-ejaculatory interval. Low levels of AME activity, such as those seen with single unit recording in the presence of sexual stimuli or upon small amounts of electrical activation, are associated with appetitive behaviors early in the reaction chain. High levels of AME activity, such as are produced by intense electrical stimulation, propel the reaction chain into the consummatory phase and yield behaviors such as the post-ejaculatory interval (Minerbo et al., 1994).

In summary, anatomical data, lesion results and findings from electrophysiology associate the AME with those neural events that subserve some aspect of the initial phase of mating behavior in male rats. A major task remaining is to determine what aspect of appetitive behavior is peculiar to the AME, which aspect is mediated specifically by the hippocampal projection of this structure and which aspects are properties of other projections of the amygdala and other limbic structures.

The place of the medial amygdala among other limbic structures in sexual behavior

Everitt (1990) has reviewed the projection of limbic, brain-stem, striatal and neocortical structures that are thought to control sexual behavior. Projections from the central and corticomedial amygdala to hypothalamus and brain-stem are thought to be involved in the visceral aspects of sexual function, the medial preoptic area of the hypothalamus (mPOA) is thought to organize a portion of the consummatory aspects of sexual performance and a projection from the ventral tegmental area to the ventral striatum is thought to produce the rewarding features of sexual activity. The basolateral, as opposed to the corticomedial, amygdala is thought to organize instrumentally conditioned behaviors in pursuit of sexual contact in this scheme.

To this circuitry we can add afferents to the amygdala from the genitalia and from olfactory structures, activation of which can drive cells in the AME (Sachs and Meisel, 1988; Murphy et al., 1996), and which may be involved in attraction and arousal. We believe that the AME is the critical focus for these cues which establish mate selection and that the projections from the AME in turn to the mPOA, bed nucleus of the stria terminalis, hippocampus and other structures are each involved in some feature of the appetitive phase of sexual behavior. Finally, we can begin to identify some of the physiological influences (excitatory, inhibitory or modulatory) and some of the likely transmitter mechanisms as discussed above and outlined in Fig. 5. Only when such mechanisms are filled in for each of the significant projections will a dynamic picture emerge of the projection pathways involved in sexual behavior.

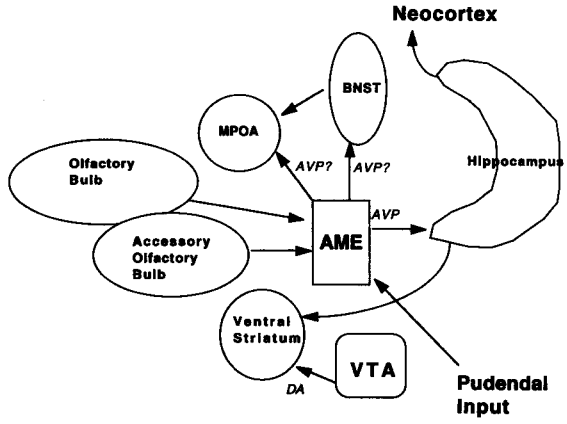

Fig. 5. A model circuit for sexual behavior in the male rat. 'DA' indicates circuit known to be mediated by dopamine, 'VP' indicates circuit known to be mediated by arginine-vasopressin or a vasopressin-like peptide, and 'VP?' indicates circuits that might be mediated by a vasopressin signal. Other abbreviations: AME, medial amygdaloid nucleus; VTA, ventral tegmental area; MPOA, medial preoptic area; BNST, bed nucleus of the stria terminalis.

Conclusions

Research on sexual behavior has lagged behind the understanding of other central phenomena such as vision, movement and plasticity. Very likely, a partial explanation for this delayed development is the fact that reproductive behavior involves the action of many steroid and peptide hormones and peptide transmitters, as opposed to simpler or conventional transmitter mechanisms, and only recently have analytical tools such as molecular probes and specific antagonists allowed us to evaluate the central action of these more complex substances and receptor mechanisms. Now, for central VP and OT, as well as for other peptide systems possibly involved in sexual behavior but not reviewed here, we can hope that burgeoning information regarding function can enable a new science of peptide-mediated processes to emerge in the field of reproductive behavior.

How might this new science contribute to problems of general interest? The fact that VP, OT and homologs are associated with sexual behavior across the phylum of chordates inspires confidence that the substances also mediate sexual behavior in primates, including humans. Apart from the intrinsic interest attached to sexual behavior in our own species, there are a number of phenomena that, if better understood mechanistically, could shed light on the circumstances and forces that gave rise to the evolution of human societies. Conspicuous among these is concealed ovulation, the tendency of some species such as bonobos (pygmy chimpanzees) and humans to

engage in sexual behavior outside of the time in which the female is ovulating (Daniels, 1983). Physical anthropologists have attached great significance to the occurrence of concealed ovulation since ignorance of the male (and possibly the female) regarding the time of peak fertility would require males to be in more constant attendance to the females throughout the cycle. Such attendance may have had adaptive significance in keeping 'fathers at home' to provide for more developmentally demanding young (Alexander and Noonan, 1988) or it may have served to prevent infanticide in promiscuous species (Hrdy, 1981). Though initially conceived as being restricted to apes (and hence a feature particularly important in hominid evolution), more recent results show that it is widespread among primates (Sillen-Tullenberg and Möller, 1993) and may be a latent capacity through the family. Of most significance is the fact that baboons (Elton and Anerson, 1977) and macaques (Wallen, 1990) can actually be observed to shift from estrus behavior typical of most mammals (sex only at time of ovulation) to sex outside of estrus (in a manner closer to concealed ovulation) depending on social pressures. It is hard to imagine that this shift would not be associated with a commensurate change in the central peptidergic structures that subserve sexual behavior. Should the change be associated with an alteration in the brain VP and OT pathways discussed in this review, then insight could be gained into the motives that formed the human family structure and are still at work today in shaping our reproductive behavior.

Acknowledgements

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CHAPTER 5.7

Voles and vasopressin: A review of molecular, cellular, and behavioral studies of pair bonding and paternal behaviors

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Several lines of evidence have implicated the neurohypophyseal peptide, vasopressin (VP), in the mediation of complex social behaviors including affiliation, aggression, juvenile recognition and parental behavior. Recent studies in microtine rodents using cellular, molecular and behavioral approaches provide additional evidence suggesting a role for VP in the formation of pair bonding and male parental care. Monogamous and promiscuous voles differ in social behaviors such as mating-induced pair bonding, selective aggression, and male parental care. Comparative studies have demonstrated that they also differ in dynamics of VP synthesis and release associated with reproduction, in the distribution pattern and regional quantity of VP receptors, and in the promoter sequence of the V_{1a} receptor gene. In monogamous prairie voles, (*Microtus ochrogaster*), brain administration of VP induces pair bonding and male parental care whereas administration of the VP antagonist diminishes these behaviors. Together, these data suggest that VP is involved in the regulation of social behaviors in monogamous voles and differences in the brain VP system may underlie species differences in behavior and life strategy in voles.

Introduction

Beginning with de Wied's 1965 report of vasopressin (VP) effects on memory, a generation of research has demonstrated that vasopressin influences behavior and cognition via actions within the central nervous system. In addition to the effects on memory (de Wied, 1977; Dantzer et al., 1988; Bluthe et al., 1990), central VP pathways have been implicated in the regulation of body temperature (Meisenberg and Simmons, 1983; Wilkinson and Kastling, 1987), blood pressure (Pittman et al., 1982), brain development (Boer et al., 1980, 1982), and social behaviors (Ferris et al., 1984; Irvin et al., 1990; Koolhaas et al., 1990; Albers et al., 1992). Virtually all of the studies on VP's central actions have used common laboratory rodents, such as rats, mice and hamsters. Although these species account for the overwhelming majority of research subjects in behavioral neuroscience, they are highly domesticated, non-selectively social, and of limited value for studying the role of VP in certain complex behaviors such as pair bonding and male parental care. In the past 5 years there have been several reports of VP's effects on social behaviors in voles. Voles are mouse-sized rodents within the genus *Microtus*. Intense field investigation has demonstrated that some American species, such as the prairie vole (*M. ochrogaster*), are monogamous and highly paternal; whereas other closely related species, such as the montane vole (*M. montanus*), are promiscuous and non-paternal. As these species differences can also be demonstrated in laboratory-bred populations of voles, these animals provide an ideal model system for comparative studies and the monogamous species permits the study of complex social beha-

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viors, such as pair bonding and paternal care. In this review, we will summarize recent findings from studies of central VP pathways and their importance for the regulation of social behavior in voles.

The microtine model

About 3% of mammals are considered monogamous, meaning that they share a nest and territory, form pair bonds, attack intruders and show high levels of paternal care (Kleiman, 1977; Dewsbury, 1987). In the laboratory and the field, prairie voles and pine voles (*M. pinetorum*) demonstrate each of these features (Table 1) (Getz et al., 1981; FitzGerald and Madison, 1983; McGuire and Novak, 1984; Carter et al., 1986; Oliveras and Novak, 1986; Shapiro and Dewsbury, 1990; Wang and Insel, 1996). In contrast, montane voles and meadow voles (*M. pennsylvanicus*) are non-monogamous. Their social structure, usually considered promiscuous, lacks pair bonding or nest sharing between males and females during the breeding season (Jannett, 1980, 1982; Madison, 1980; Gruder-Adams and Getz, 1985; Shapiro and Dewsbury, 1990; Insel et al., 1995). The female is the lone provider of parental care (McGuire and Novak, 1984, 1986). In contrast to the monogamous species that inhabit multigenerational, communal burrows, montane or meadow voles are generally found in isolated burrows (Madison, 1980; Jannett, 1982). Species differences in social behavior can be observed even in neonatal voles: infant prairie voles respond to social separation as a stressor whereas infant montane voles show no evident behavioral or endocrine response to social separation (Shapiro and Insel, 1990).

In all of these vole species, mating is a prolonged affair with multiple copulatory bouts over a 24 h period. The behavioral consequences of mating are markedly different in monogamous and non-monogamous voles. In the prairie vole, for instance, 24 h of mating establishes a pair bond between a male and a female, evident by the formation of a preference for the mate versus a conspecific stranger and the emergence of aggression towards intruders into the nest (Carter et al., 1986; Shapiro and Dewsbury, 1990; Carter and Getz, 1993; Winslow et al., 1993; Insel et al., 1995). The partner preference and nest guarding persist for at least 2 weeks even in the absence of further exposure to the mate (Winslow et al., 1993; Insel et al., 1995). After parturition, both parents display intense parental care (McGuire and Novak, 1984; Oliveras and Novak, 1986). On the other hand, non-monogamous voles, such as montane or meadow voles, show little interest in social contact, mating does not induce a pair bond and aggression, and males display little if any parental care (McGuire and Novak, 1986; Oliveras and Novak, 1986; Shapiro and Dewsbury, 1990; Insel et al., 1995).

At least three aspects make voles particularly useful for the study of VP. First, the profound differences in pair bond and parental behavior between vole species provide an intriguing natural experiment for studying neuroanatomic features (e.g. VP pathways) associated with evolved patterns of social behaviors. Second, the development of social behaviors triggered by reproductive events such as mating or parturition in the monogamous species, permit the study of neural mechanisms of pair bonding. Studies in the prairie vole might indicate if reproductive behavior influences VP or if VP influences pair bonding and parental care. Finally, sexual dimorphism tends to be mini-

Table 1
Social organization and behaviors in voles

	Praire (*M. ochrogaster*)	Pine (*M. pinetorum*)	Montane (*M. montanus*)	Meadow (*M. pennsylvanicus*)
Life strategy	Monogamous	Monogamous	Promiscuous	Promiscuous
Pair bond	Yes	?	No	?
Nest sharing	Yes	Yes	No	No
Parental	Biparental	Biparental	Maternal	Maternal

mal in monogamous species (Emlen and Oring, 1977; Shapiro et al., 1991). Vasopressin has been previously shown to be highly sexually dimorphic in the rat brain (De Vries, 1990). Study of monogamous voles, therefore, might indicate if sexual dimorphisms persist in the brains of rodents when males and females perform similar social roles.

Diversity of central vasopressin systems in voles

We have used the comparative approach to determine if voles with different social organization and behavior show differences in VP cells or projections, VP receptor distribution or VP receptor gene sequence. These studies asked (a) how do monogamous species differ from non-monogamous species? and (b) how do males and females differ within the monogamous species?

Vasopressin cells and fibers

Central VP immunoreactivity and mRNA expression have been examined in a variety of vole species (Bamshad et al., 1993; Wang et al., 1994b, 1996; Wang, 1995). VP immunoreactive (VP-ir) or VP mRNA-labeled cells are found in dense clusters in the paraventricular (PVN), supraoptic (SON) and, to a lesser extent, in the suprachiasmatic (SCN) nucleus of the hypothalamus. Scattered VP-ir or VP mRNA-labeled cells are found in extrahypothalamic areas such as the bed nucleus of the stria terminalis (BST) and medial nucleus of the amygdala (MA). VP-ir fibers are found in several brain areas such as the lateral septum (LS), lateral habenular nucleus (LH), diagonal band (DB), medial preoptic area (MPO), BST, PVN and the MA. The morphology and distribution pattern of these VP cells and fibers are not only similar among voles that have different social organization and behaviors, but also resemble those found in other species of rodents (Buijs et al., 1978; De Vries et al., 1985; Mayes et al., 1988; Hermes et al., 1990; Bittman et al., 1991).

A sexually dimorphic pattern is evident for VP pathways in voles. Contrary to prediction, marked sexual dimorphisms are evident in both monogamous and non-monogamous voles. Across four species (prairie, pine, montane and meadow), males have more VP-ir or VP mRNA-labeled cells in the BST, and a higher density of VP-ir fibers in the LS and LH than females (Fig. 1; Bamshad et al., 1993; Wang et al., 1994b, 1996). This sexually dimorphic VP pathway is steroid dependent: castration reduces the number of VP-ir cells in the BST and MA as well as the density of VP-ir fibers in the LS and LH, whereas testosterone replacement restores the VP-ir staining of castrated males to the level of intact male voles (Fig. 2; Wang and De Vries, 1993). This gender dimorphism and steroid dependence has been previously reported in several species of rodents such as rats, mice, hamsters and gerbils (van Leeuwen et al., 1985; Mayes et al., 1988; Hermes et al., 1990; Bittman et al., 1991; Crenshaw et al., 1992). Castration and testosterone treatment do not induce significant changes in VP expression in the hypothalamic nuclei (PVN, SON and SCN) in voles (Wang and De Vries, 1993; Wang, Young and Insel, unpublished data) nor in other species of rodents (Crowley and Amico, 1993). In spite of marked differences in behavior, the morphology, distribution, and characteristics suggest that VP synthesizing cells and fibers are similar across voles and, in general, voles resemble pathways reported in other species of rodents.

Vasopressin receptor distribution

The actions of VP are mediated by three subtypes of membrane-bound receptors, namely V_{1a}, V_{1b} and V_2 receptors, with V_{1a} predominating in the central nervous system (Jard, 1983; Van Leeuwen et al., 1987; Barberis and Tribollet, 1996). Voles with different social organization and behavior differ in their brain VP receptor distribution. Studies using tritiated (^{3}H-VP) and iodinated VP ligands (^{125}I-sarc-VP) with in vitro receptor autoradiography have demonstrated that monogamous prairie and pine voles show a similar pattern of VP receptor binding, with little overlap with the receptor distribution observed in promiscuous montane and meadow voles (Insel et al., 1994). In addition, male and female voles show a similar distribution pattern of VP receptor binding within each species. These data suggest that species differences in the distribution pattern of central VP receptors appear to be

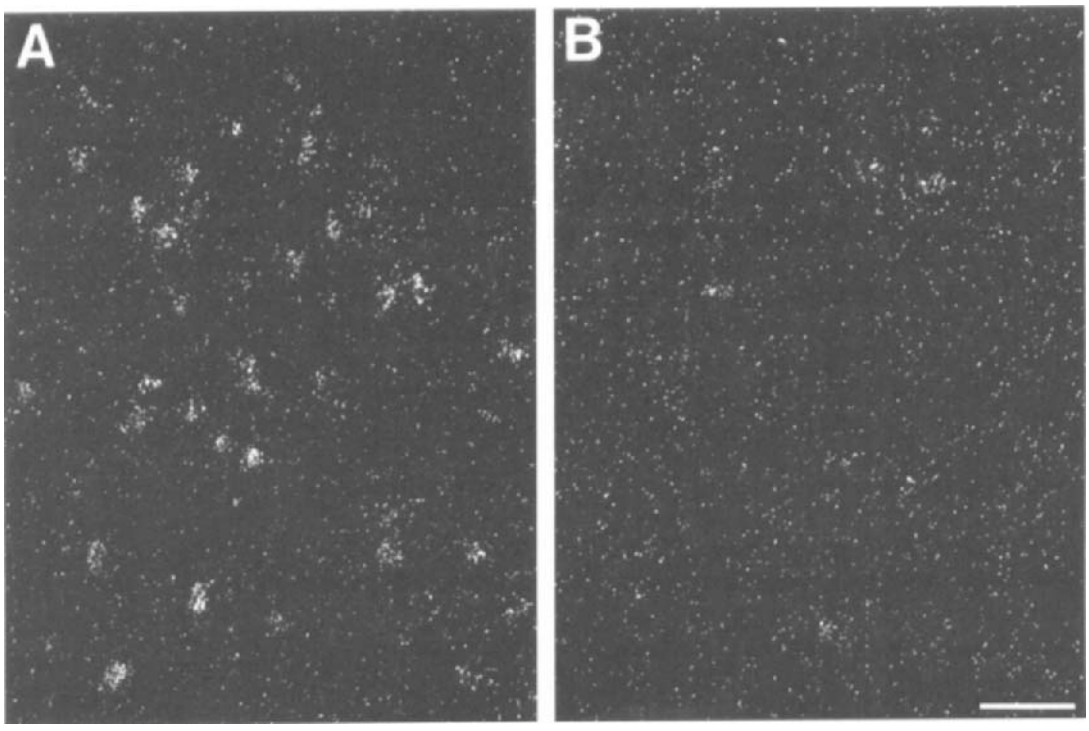


Fig. 1. Photomicrographs of dark-field-illuminated sections displaying cells labeled for VP mRNA in the bed nucleus of the stria terminalis in male (A) and female (B) prairie voles. Scale bar, 50 mm (adapted from Wang et al., 1994b).

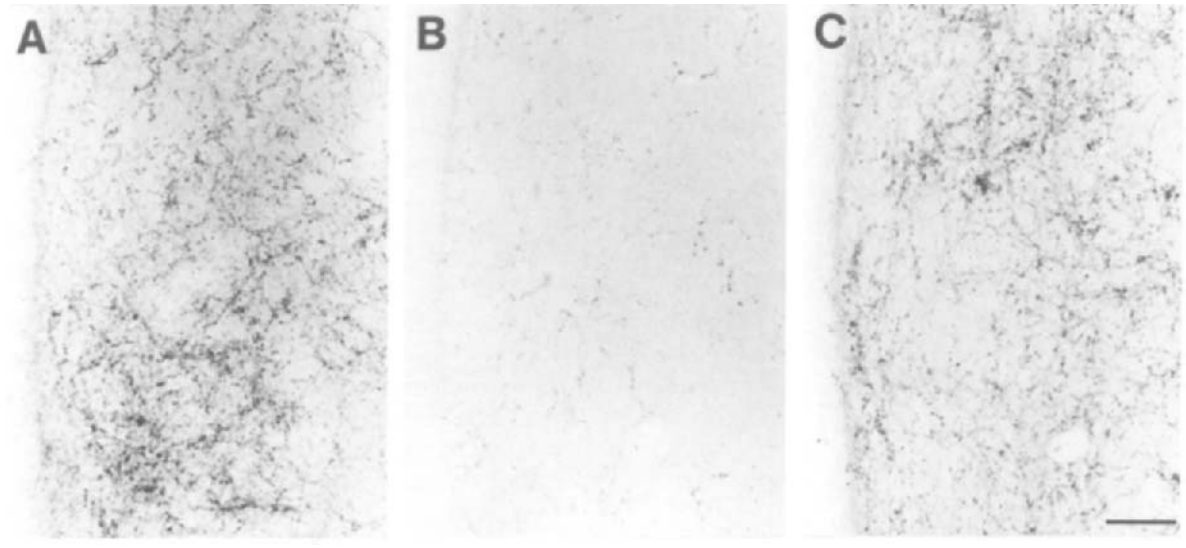


Fig. 2. Photomicrographs displaying VP-ir fiber plexus in the lateral septum of male prairie voles that were sham-operated (A), castrated for 4 weeks (B), or castrated and implanted with testosterone (C). Scale bar, 50 mm (adapted from Wang and De Vries, 1993).

associated with the pattern of social organization in voles. In a recent study using newly developed ^{125}I-linear-VP ligand (Johnson et al., 1993; Barberis et al., 1995), VP receptor binding was identified in anatomically matched coronal sections from prairie and montane voles (Fig. 3; Wang et al., 1997). Prairie voles have a higher density of VP receptors labeled by ^{125}I-linear-VP in the diagonal band, central nucleus of the amygdala, cingulate cortex and laterodorsal thalamus than montane voles. On the other hand, montane voles have more VP receptor binding in the lateral septum, ventroposterior and reticular thalamus than prairie voles.

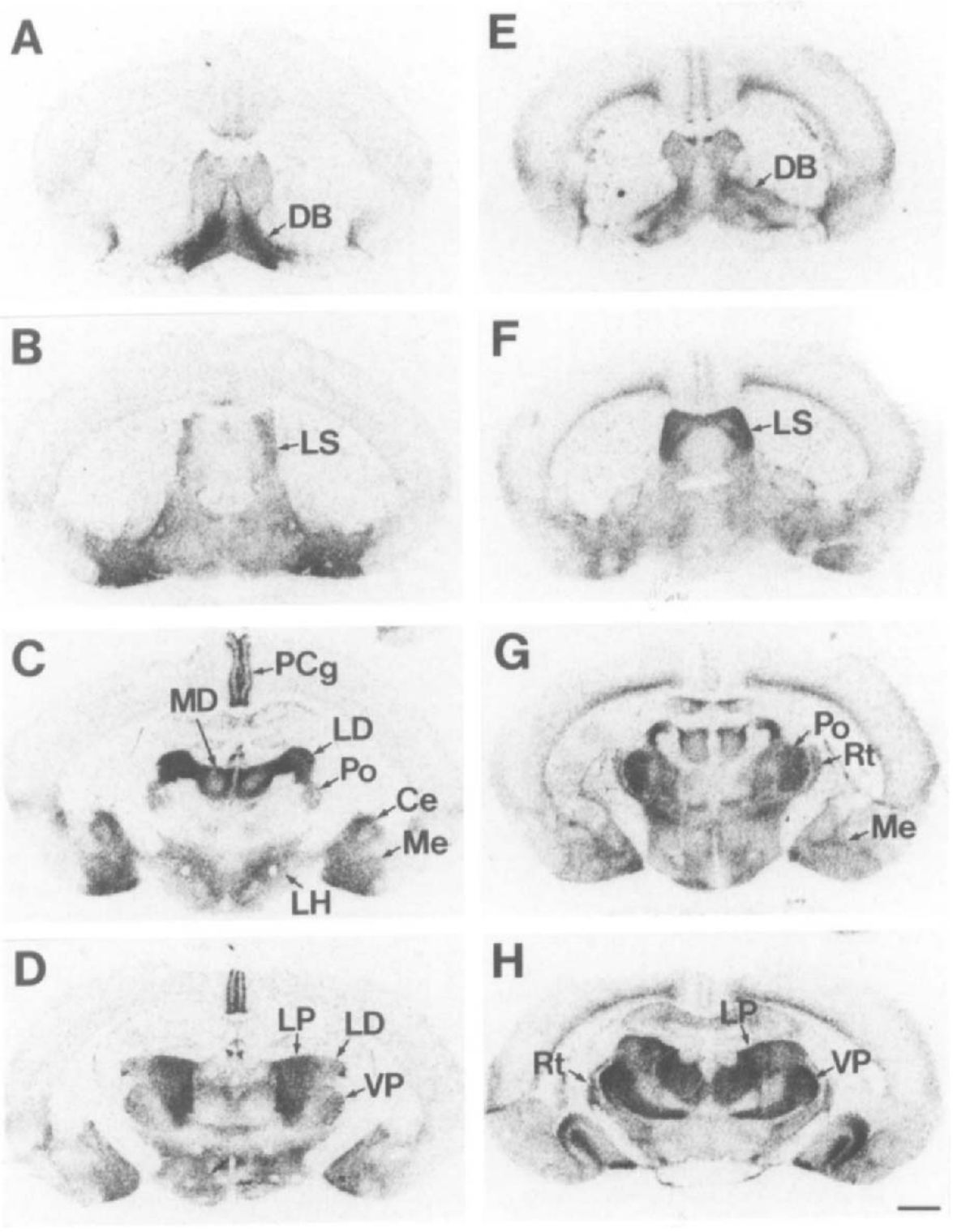
We have recently cloned and sequenced the V_{1a} receptors in both prairie and montane voles (Young et al., 1997). Our results indicate that both species share the same receptor (see below), and in situ hybridization with a single cRNA probe identifies a pattern of VP receptor mRNA in each species that resembles the distribution pattern of binding (Fig. 4). Together, these data suggest that (1) voles with different social organization have different patterns of VP receptor distribution in the brain, (2) differences in VP receptor binding represent differences in localization of a single receptor and do not result from a promiscuous ligand binding to two different receptors, and (3) functional responses to VP might be very different in these species as the peptide influences a different set of targets in monogamous versus non-monogamous voles.

In a previous study, we reported that prairie and pine voles show a similar distribution pattern of brain oxytocin (OT) receptors which differs from that of montane and meadow voles (Insel and Shapiro, 1992). It does not appear, however, that voles show unusual species variability in the expression of all membrane-bound receptors in the brain, as comparative studies across four vole species found no differences in the distribution of mu opiate or benzodiazepine receptors (Insel and Shapiro, 1992).

Species differences in receptor distribution could arise from a common pattern in development with the two species diverging at a critical point such as weaning or puberty. Alternatively, vole species could differ throughout development. Studies of the ontogeny of ^{125}I-linear-VP binding show species differences even in the perinatal period (Fig. 5; Wang et al., 1997). For instance, in the BST and central amygdala, prairie voles have higher ^{125}I-linear-VP binding at birth than montane voles. This difference, despite its variations during early development, is sustained at weaning and into adulthood, indicating that species differences in VP receptor binding are probably determined by prenatal and/or genetic factors. In the lateral septum, species differences in VP receptor binding arise postnatally: binding increases rapidly in montane voles but remains unchanged in prairie voles. VP receptors in the cingulate cortex in prairie voles show dense binding in early development followed by a rapid decrease to reach the adult level at weaning: a developmental pattern that has been observed in rats (Snijdewint et al., 1989; Tribollet et al., 1991). In summary, as in the rat brain, VP receptor binding in the vole brain shows early appearance, transient expression, and redistribution over the course of postnatal development.

Vasopressin receptor gene sequence

As mentioned above, the striking differences in vasopressin receptor distribution between the monogamous prairie vole and the promiscuous montane vole appear to be the result of species differences in V_{1a} receptor gene expression in the brain. We have begun to investigate the molecular mechanisms which could be responsible for these species differences. Since tissue specific regulation of gene expression is typically regulated by specific *cis* regulatory sequences located in the 5′ flanking region, we hypothesized that the species differences in regional V_{1a} receptor gene expression could be due to differences in the 5′ flanking region of the V_{1a} gene. We have cloned and sequenced the V_{1a} receptor gene from both prairie and montane voles. The structure and sequence homology of the receptor genes is illustrated in Fig. 6. The coding region of the gene is virtually identical between these two species. However, preliminary analysis of the 5′ flanking region of the V_{1a} receptor gene reveals striking species differences. The first 1000 bp of the promoter of both vole species share moderate sequence homology with the rat V_{1a} receptor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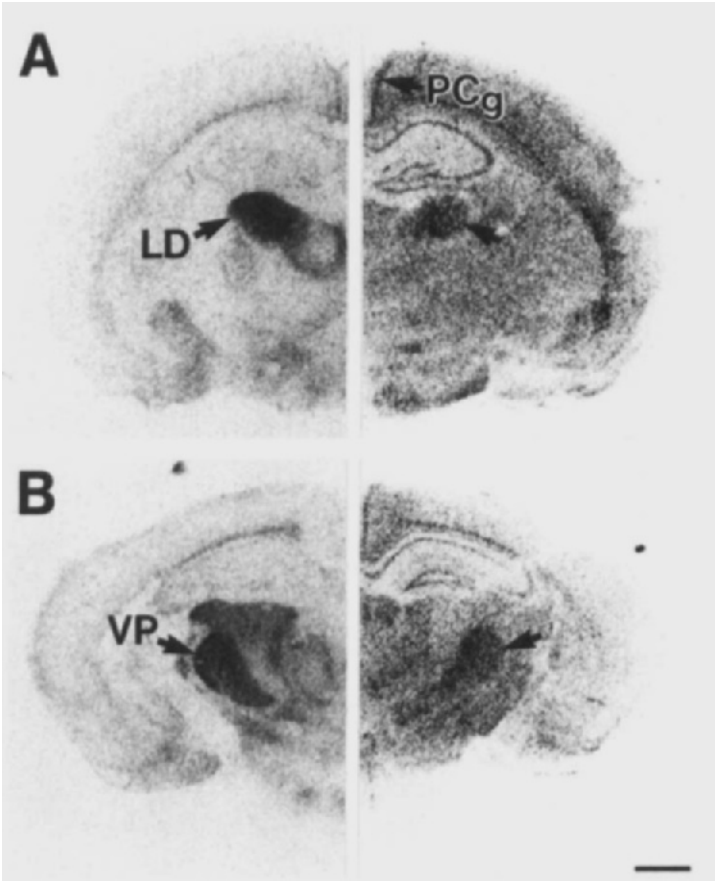

Fig. 4. Photomicrographs displaying VP receptor binding (left) and mRNA labeling (right) on the brain section of a prairie (A) and a montane vole (B). Scale bar, 1 mm (adapted from Young et al., 1997).

gene (Murasawa et al., 1995), and high homology with each other. However, a sudden complete loss of sequence homology is found beyond 1000 bp. Comparison of this region with the rat V_{1a} receptor gene reveals significant homology between the rat sequence and the montane vole sequence. This sequence homology between the montane vole and rat might indicate that the montane vole V_{1a} receptor gene is the ancestral form, while the prairie vole gene is the derived form. This hypothesis is also supported by the similarity in V_{1a} receptor binding pattern in the rat and montane vole. The sequence data suggest that the promoter of the prairie vole V_{1a} receptor gene, or that of a recent ancestor, has been modified, most likely due to translocation or an insertional event. This event may be related to the species difference in V_{1a} receptor gene expression, and potentially to the evolution of social behavior in the prairie vole. We are currently investigating this hypothesis by analyzing the promoters of other vole

Fig. 3. Photomicrographs displaying ^{125}I-linear-VP binding in paired coronal sections from a prairie vole (A–D) and a montane vole (E–H). Ce, central amygdala; DB, diagonal band; LD, laterodorsal thalamus; LH, laterodorsal hypothalamus; LP, lateroposterior thalamus; LS, lateral septum; MD, mediodorsal thalamus; Me, medial amygdala; PCg, cingulate cortex; Po, posterior thalamus; Rt, reticular thalamus; VP, ventroposterior thalamus. Scale bar, 1 mm (adapted from Wang et al., 1997).

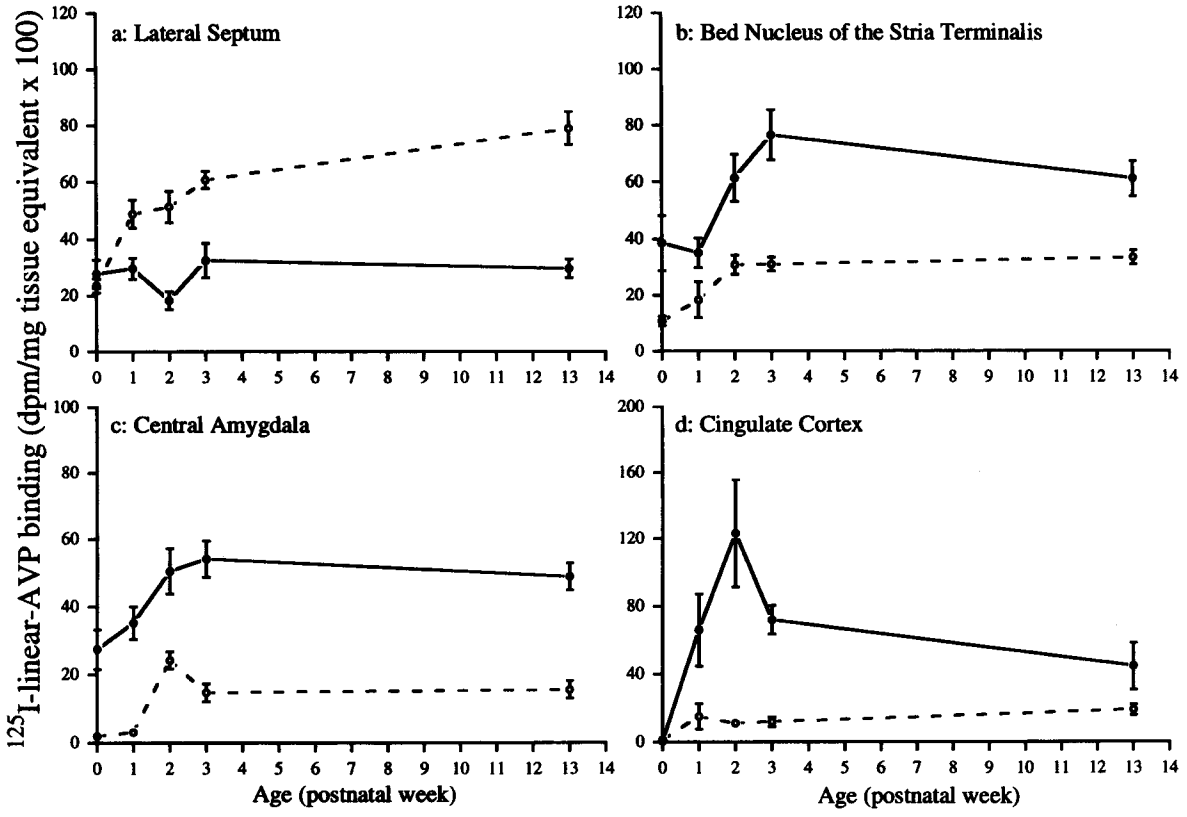

Fig. 5. Ontogeny of ^{125}I-linear-VP binding in the lateral septum (a), bed nucleus of the stria terminalis (b), central nucleus of the amygdala (c) and cingulate cortex (d) in prairie voles (●) and montane voles (○). Each point indicates a mean ± SEM from five to six animals (adapted from Wang et al., 1997).

species with similar differences in receptor distribution and social behavior.

Changes of central vasopressin associated with reproductive behavior

Voles with different social organization and behaviors show differences not only in their VP receptor distribution, but also in the dynamics of central VP release and gene expression. We have investigated changes of central VP activity during mating and reproduction in both monogamous and promiscuous voles.

Mating-induced changes in vasopressin activity

Mating induces a behavioral transformation in prairie voles. After 24 h of mating, male and female prairie voles exhibit affiliative behavior only towards their mates, and males attack conspecific strangers (Shapiro and Dewsbury, 1990; Winslow et al., 1993; Insel et al., 1995). Both affiliation and aggression appear to be dependent on mating as cohabitation with a female without mating does not induce such behaviors (Winslow et al., 1993; Insel et al., 1995). Furthermore, the emergence of affiliation and aggression in prairie voles appears to represent the development of a pair bond as non-monogamous voles fail to show these behaviors after mating (Shapiro and Dewsbury, 1990; Insel et al., 1995).

In addition to effects on behavior, mating also induces changes in extra-hypothalamic VP activity in the vole brain in a sex- and species-dependent

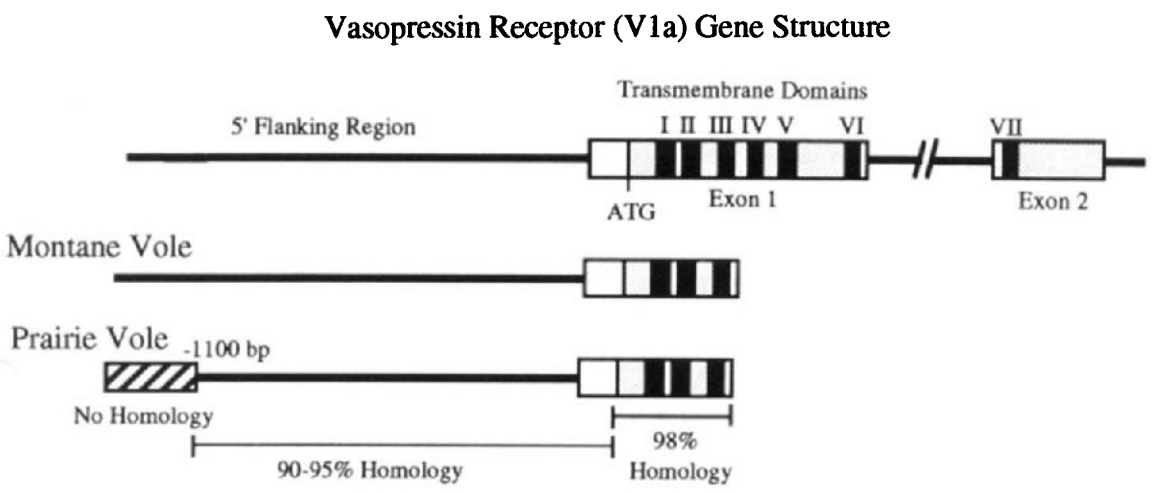

Fig. 6. The sequence homology of the 5′ end of montane vole and prairie vole V_{1a} receptor clones is illustrated below the a schematic of the full length V_{1a} receptor gene. The coding sequences share 98% homology while the first 1100 bp of the 5′ flanking region share 90–95% sequence homology between the vole species. At −1100 bp the sequence homology abruptly ends (stripped box). The montane vole sequence in this region continues to show homology with the rat gene sequence suggesting that the prairie vole sequence has been modified.

manner. After 3 days of mating and cohabitation with a female, male prairie voles have a reduced density of VP-ir fibers in the lateral septum and an enhanced level of VP mRNA expression in the BST relative to sexually naive males (Fig. 7; Bamshad et al., 1994; Wang et al., 1994b). The enhanced VP mRNA expression in the BST is associated with increased plasma testosterone (Wang et al., 1994b). In rodents, VP cells in the BST project to the lateral septum (De Vries and Buijs, 1983; De

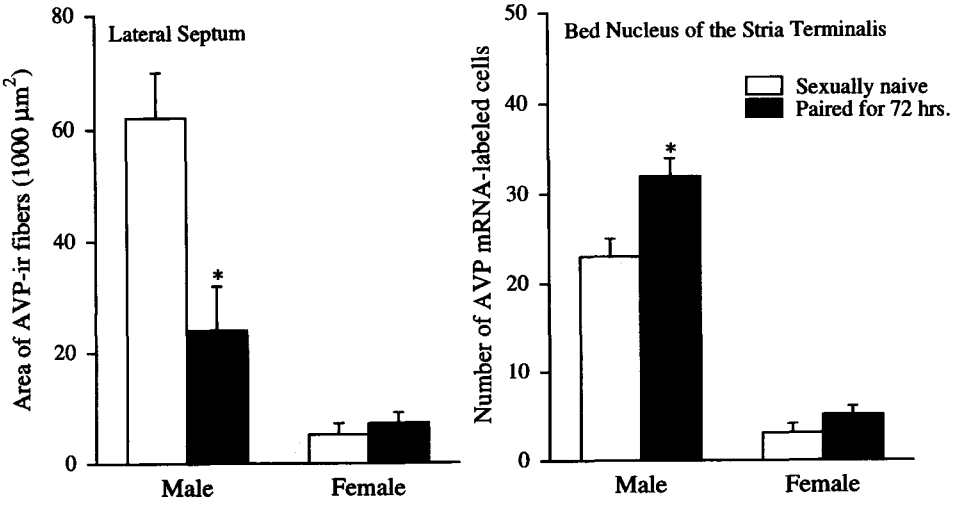

Fig. 7. Effects of 3 days mating and cohabitation on the density of VP-ir fibers in the lateral septum (left) and the number of VP mRNA-labeled cells in the bed nucleus of the stria terminalis (right) in male and female prairie voles. Scale bars, mean ± SEM; $*P < 0.05$ (adapted from Bamshad et al., 1994 and Wang et al., 1994b).

Vries et al., 1985). Therefore, decreased VP-ir staining in terminals along with increased VP synthesis in the cell bodies probably suggests that mating induces septal VP release in male prairie voles. Such mating effects on central VP activity are not found in female prairie voles nor are they found in either sex of non-monogamous meadow voles, suggesting that mating-induced septal VP release is male-specific and restricted to the monogamous vole (Bamshad et al., 1994; Wang et al., 1994b).

In a recent experiment, we found that mating did not alter VP mRNA expression in the hypothalamic nuclei (PVN and SON). In addition, we could not detect a mating-induced change in brain VP receptor binding in either male or female prairie voles (Wang, Young and Insel, unpublished data). Taken together, these data suggest that in male prairie voles, mating induces hormonal changes which, in turn, trigger central VP release in a specific brain area (e.g. the lateral septum). The released VP may play a role in the regulation of social behaviors such as partner preference and aggression in prairie voles, as it does in social recognition and aggression in other rodents (Le Moal et al., 1987; Dantzer et al., 1988; Compaan et al., 1993). That mating-increased testosterone has no effects on VP mRNA expression in the PVN and SON in male prairie voles is in agreement with the finding in rats, in which castration and testosterone treatment do not induce changes in VP mRNA expression in these nuclei (Crowley and Amico, 1993). In non-monogamous voles, the lack of mating-induced central VP release (testosterone was not increased by mating in non-monogamous meadow voles, (Wang et al., 1994b)) as well as different central target sites of VP actions may at least partially account for the absence of mating-induced changes in their social behaviors.

Changes associated with parturition

Species differences in parental behavior are also associated with differences in extra-hypothalamic VP activity. With immunocytochemical staining, parental male prairie voles (at postpartum day 6) have a significantly reduced density of VP-ir fibers in the lateral septum and lateral habenular nucleus relative to their sexually naive counterparts (Fig. 8; Bamshad et al., 1993). In contrast, meadow vole fathers at postpartum day 6 do not show parental behavior to their young and have a level of VP-ir staining in both brain areas equivalent to that of sexually naive males (Fig. 8). No changes in VP-ir staining are found in females of either species. Although it is not yet clear that parental and sexu-

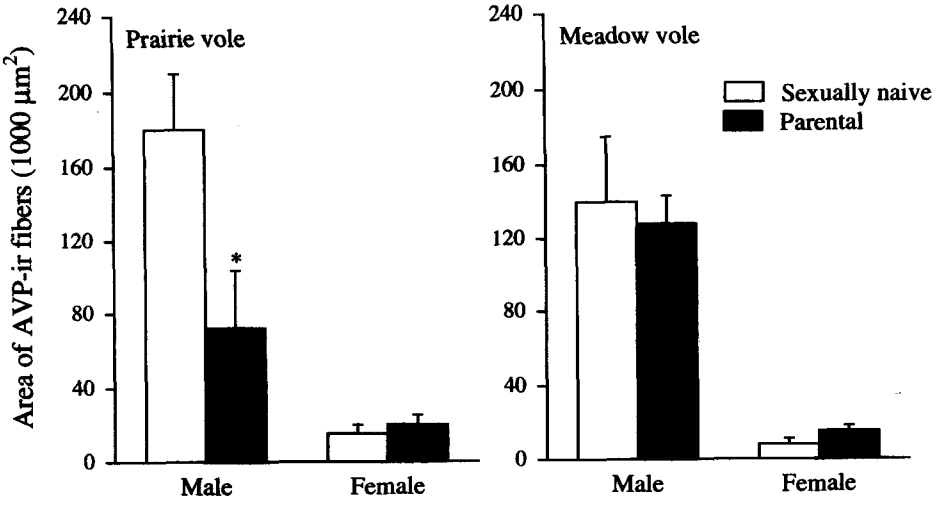

Fig. 8. Differences in the density of VP-ir fibers in the lateral septum of sexually naive (□) and parental (at postpartum day 6; ■) male and female prairie and meadow voles. Scale bars, mean ± SEM; $*P < 0.05$ (adapted from Bamshad et al., 1993).

ally naive male prairie voles have different levels of VP mRNA expression in the BST, reduced VP-ir staining in the terminal fields (in the lateral septum and lateral habenular nucleus) may reflect increased VP release. It appears, therefore, that both mating and paternal care may be associated with extra-hypothalamic VP release in monogamous male voles.

The effects of reproduction on VP activity are not limited to the sexually dimorphic pathways. Reproduction is also found to influence VP mRNA expression in the hypothalamic nuclei. Female prairie voles at postpartum day 1 and 6 have increased VP mRNA (Fig. 9) as well as OT mRNA expression in the PVN and SON relative to sexually naive females. These data resemble

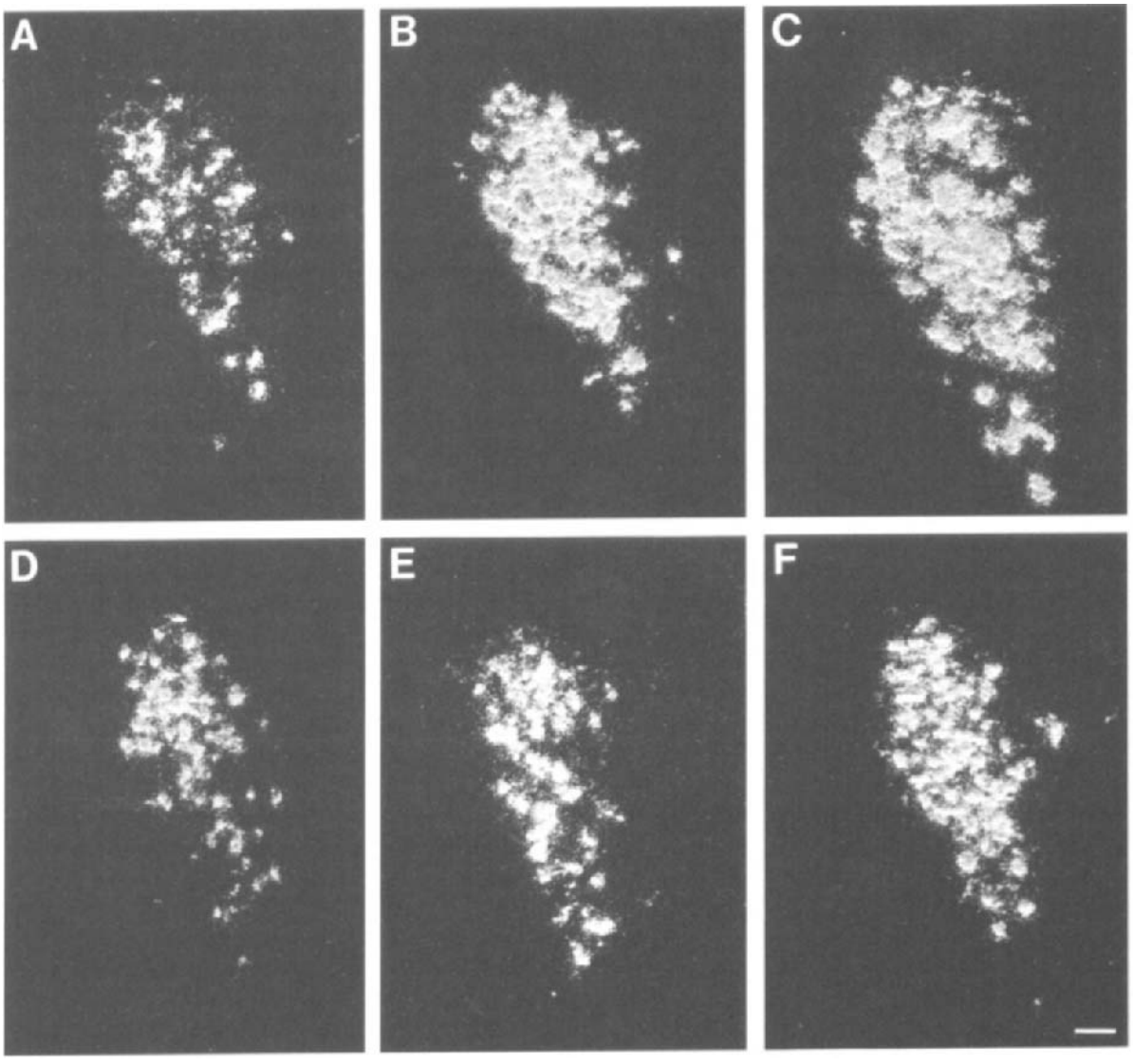

Fig. 9. Photomicrographs of dark-field-illuminated sections displaying VP mRNA-labeled cells in the paraventricular nucleus of the hypothalamus of male (A–C) and female (D–F) prairie voles. Sexually naive voles (A and D) had less VP mRNA expression in the PVN than voles at day of litter birth (postpartum day 1; B and E) or at postpartum day 6 (C and F). Scale bar, 50 mm.

results from female rats in which pregnancy, parturition and lactation are associated with a parallel elevation of VP and OT mRNA expression in the PVN and SON (van Tol et al., 1988; Zingg and Lefebvre, 1988), apparently due to sequential changes of gonadal steroids associated with pregnancy and parturition (Crowley et al., 1995; Thomas et al., 1995, 1996). Increased OT is related to uterine contraction at parturition, milk ejection in response to suckling and maternal behavior, whereas increased VP may be involved in uterine contraction and maintaining homeothermy and other autonomic events associated with female reproduction (Pedersen et al., 1982; Higuchi et al., 1986; Caldwell et al., 1987).

Interestingly, male prairie voles at postpartum day 1 and 6 also show an increase in VP mRNA expression in the PVN and SON relative to sexually naive males (Fig. 9). No postpartum effects, however, are detected in their OT mRNA expression. The reason for increased VP mRNA-labeling in the PVN and SON in male prairie voles is still unknown. While VP may influence the emergence of paternal behavior, it is also possible that paternal behavior influences VP gene expression. For instance, one aspect of parental behavior is anogenital licking of pups, including the ingestion of hypertonic urine (Baverstock and Green, 1975; Friedman and Bruno, 1976). As osmotic challenges increase hypothalamic VP mRNA levels (Zingg et al., 1986; Crowley et al., 1993), the increase in VP mRNA expression in male prairie voles may simply reflect urine ingestion (Bamshad et al., 1993). Non-monogamous montane or meadow voles may not show the increase in hypothalamic VP mRNA expression since they do not display paternal behavior.

Vasopressin regulation of social behavior

The above-mentioned neuroanatomical and neurochemical studies indicate species differences in VP systems in monogamous and non-monogamous voles. These studies provide correlational evidence but they fail to demonstrate that central VP is involved in social behavior in voles. Recent pharmacological studies provide direct evidence that central VP may be critical for social behavior in monogamous voles.

Involvement of central vasopressin in pair bonding

If mating facilitates pair bond formation and VP is released with mating, does VP influence the development of a pair bond? To answer this question, two aspects of pair bonding, mating-induced aggression and partner preference formation, have been studied in prairie voles (Winslow et al., 1993). Males receiving central injections of CSF or an OT antagonist exhibit aggression after 24 h of mating. However, males that receive an injection of a V_{1a} antagonist, ($d(CH_2)_5$[Tyr(Me)]VP), do not show mating-induced aggression (Fig. 10). This effect on aggression cannot be attributed to effects on mating, as sexual behavior is not influenced by the V_{1a} antagonist. In addition, the V_{1a} antagonist does not seem to be anti-aggressive per se because breeder males with established aggression show no decrease in aggression 1 or 24 h after receiving the V_{1a} antagonist injection (Winslow et al., 1993). These data suggest that the VP antagonist blocks the transition to aggression, not its expression in male prairie voles.

As noted above, following mating, male prairie

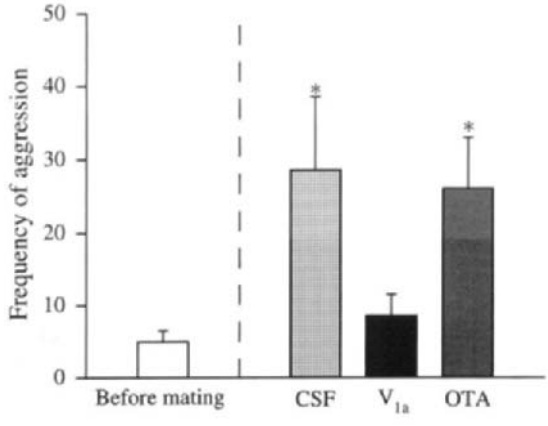

Fig. 10. Differences in male aggression after 24 h mating. Males receiving injections of a V_{1a} antagonist (5 ng) did not show mating-induced aggression whereas males receiving injections of either CSF or OTA (5 ng) showed mating-induced aggression. Scale bars, mean ± SEM; $*P < 0.05$ (adapted from Winslow et al., 1993).

voles demonstrate a partner preference, an early manifestation of pair bonding. Males receiving an infusion of CSF or OT antagonist exhibit mating-induced partner preferences whereas males receiving a V_{1a} antagonist do not develop a partner preference, suggesting an involvement of VP in partner preference formation (Fig. 11a).

It appears that VP may be not only necessary but also sufficient for pair bond formation. Twenty-four hours of cohabitation with a female without mating does not induce a partner preference in male prairie voles. However, if males receive a central VP infusion, they exhibit a preference for a female partner even in the absence of mating (Fig. 11b; Winslow et al., 1993). VP infusions also induce aggression in male prairie voles. These effects appear specific, as OT at the same dose (0.5 ng/h) does not facilitate either partner preference formation or aggression in males. Curiously, central infusions of OT facilitated and infusions of the OT antagonist diminished partner preference formation in female prairie voles (Williams et al., 1994; Insel and Hulihan, 1995). In a recent study, a single injection of a high dose of VP or OT (100 ng) stimulated partner preference formation in both male and female prairie voles, but the temporal profiles of the peptide's effects need to be studied further (Cho et al., 1996).

Vasopressin regulation in male parental care

Mating with a female not only induces a pair bond but also enhances paternal responsiveness to a conspecific pup (Bamshad et al., 1994). This enhanced paternal responsiveness is associated with changes in central VP-ir staining and mRNA expression (Bamshad et al., 1994; Wang et al., 1994b). Male prairie voles that receive a septal VP injection exhibit a high level of paternal behavior and this VP effect is blocked by a pre-injection of a V_{1a} antagonist (Fig. 12a). In addition, males injected with the V_{1a} antagonist exhibited a lower level of paternal behavior than males injected with saline (Fig. 12b). Combined, these data suggest that VP acts on the V_{1a} receptors to influence male parental behavior in prairie voles (Wang et al., 1994a). Central VP pathways have been previously implicated in parental behavior in other rodents. Long–Evans rats, for example, display superior parental behavior in comparison with the VP-deficient mutant Brattleboro rats (Wideman and

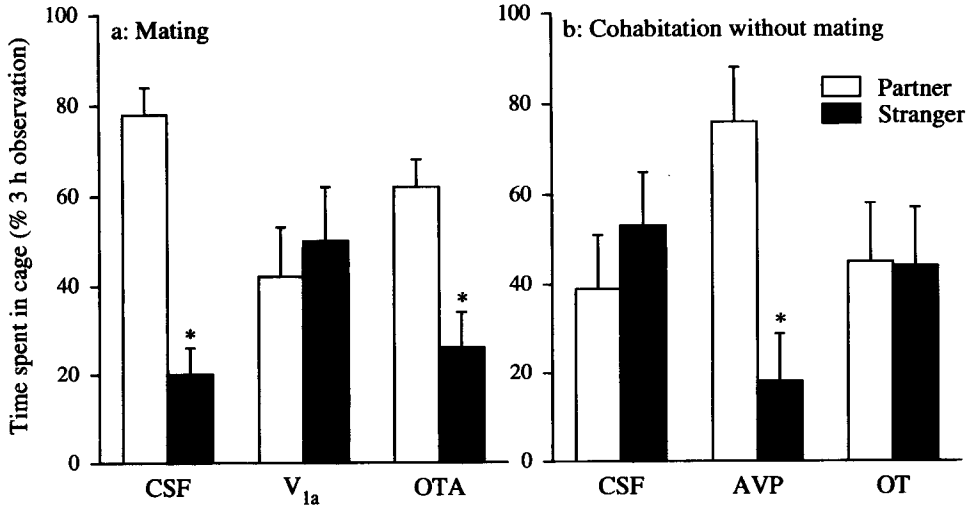

Fig. 11. Differences in preference of a partner versus strangers in male prairie voles that were either mated for 24 h (left) or cohabited with a female for 24 h without mating (right). Central injections of a V_{1a} antagonist (5 ng) diminished mating-induced partner preference whereas infusions of VP (0.5 ng/h) induced partner preference in the absence of mating. Scale bars, mean ± SEM; $*P < 0.05$ (adapted from Winslow et al., 1993).

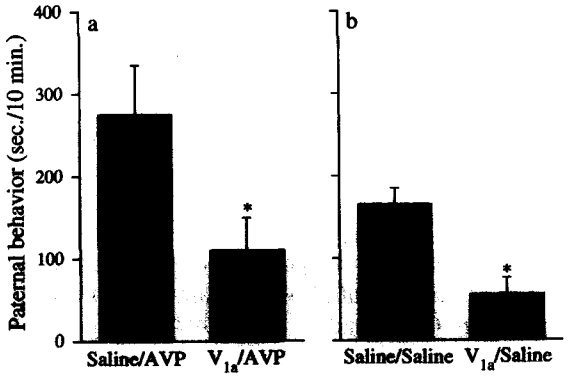

Fig. 12. Differences in paternal behavior between male prairie voles that received an injection of saline followed by an injection of VP (0.1 ng/100 nl saline) or a V_{1a} antagonist (1 ng/100 nl saline) followed by VP (a), or an injection of saline followed by saline or a V_{1a} antagonist followed by saline (b). Scale bars, mean ± SEM; $*P < 0.05$ (t test) (adapted from Wang et al., 1994a).

Murphy, 1990). Injections of VP into the lateral ventricle induces persistent parental behavior in female rats (Pedersen et al., 1982). It is not clear whether VP plays a role in initiation or maintenance of parental behavior in female prairie voles or non-monogamous voles of either sex.

Conclusions

Voles show remarkable differences in social behaviors associated with life strategy. Monogamous voles exhibit mating-induced pair bonding and biparental care, whereas promiscuous voles show neither pair bonding nor male parental care. These animals, thus, provide an ideal model system for the comparative studies of the neurobiological basis of social behavior. For example, by studying monogamous voles that exhibit remarkable behavioral changes during reproduction, one can explore neural mechanisms underlying changes of social behavior as well as the influence of complex behavior on the central nervous system. Although such approaches are still very recent, data from several studies have demonstrated the importance of VP in the regulation of social behaviors in voles.

Voles with different life strategy and behaviors show different distribution patterns of brain V_{1a} receptors, indicating differences in neural target sites for VP. This difference in receptor distribution could be due to species variation in the promoter sequences of the V_{1a} receptor gene. Monogamous and promiscuous voles also differ in the patterns of VP synthesis and release associated with reproduction. Mating in monogamous male voles induces an increase in extra-hypothalamic VP release and this released VP has been implicated in pair bonding and enhanced paternal care. Central injection/infusion of VP enhances pair bonding and male parental care whereas injections of the V_{1a} antagonist diminish these behaviors. Reproduction does not appear to influence central VP activity or social behaviors in promiscuous male voles. Finally, VP gene expression in the PVN and SON in male prairie voles does not change after mating but increases significantly with the onset of paternal care. Together these data suggest a dynamic mechanism by which VP regulates behavioral and physiological functions. On one hand, VP synthesis can be triggered by reproductive events and VP is released into specific central areas to regulate behaviors associated with reproduction. On the other hand, VP synthesis can also be increased in response to the altered physiological demands of social behavior. VP is, then, released peripherally or centrally to regulate physiological functions. Although studies on VP in voles are still at an early stage, the data, so far, have demonstrated voles as a useful model system for studying natural selection in social behavior, VP, and their interactions.

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CHAPTER 5.8

Role of vasopressin in learning and memory in the hippocampus

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The involvement of arginine^8-vasopressin (VP) in learning and memory in the hippocampus is examined in mice using a discriminative learning task. Bilateral dorsal hippocampal lesion blocks the enhancing effect of intracerebroventricular (i.c.v.) injection of VP on retrieval and relearning processes. An additional study showed that immunoneutralization of dorsal hippocampal endogenous VP inhibited the facilitating effect of i.c.v. injection of VP, suggesting that hippocampus is essential for the expression of VP's behavioral effects. Using in situ microinjection, a greater sensitivity of the ventral part of the hippocampus to the memory enhancing effects of VP has been reported. This effect is mediated by vasopressin V1 type receptors and oxytocin receptors. Then, we examined the effects on behavior of VP applied to the ventral hippocampus, in relation to the time of treatment during learning. When the animals have no previous information about the task to learn, a deleterious effect of VP appears (pre-first session treatment). Regarding memory consolidation, the effects of VP may depend upon the previous level of performance acquired by the animals since, when injected after the first learning session, the peptide slightly delayed performance, whereas when the injection took place after the second learning session, it enhanced learning. Concerning memory retrieval, the effects of VP depend on the quality of the previously stored information. The fact that VP did not generate the same behavioral effects when the treatment was performed at the beginning or in the middle of the learning processes, suggests that mnemonic context is an important factor in understanding the effect of VP on memory in the ventral hippocampus. Finally, the role of hippocampal adrenergic receptors in the enhancing VP effects on memory retrieval has been examined. The facilitatory effects of VP seem to depend upon the functional state of both alpha- and beta-adrenergic receptors, but further studies will be necessary to clarify the role played by each receptor type in retrieval processes, and to determine the relationships that might exist between them.

Introduction

In addition to its endocrine and numerous physiological functions, arginine^8-vasopressin (VP), one of the two post-hypophysial peptides, plays a role in learned behaviors. Following the pioneering work by De Wied nearly 20 years ago, many studies have contributed to improving our understanding of the involvement of VP in learning and memory processes. The central target structures and the receptors implicated in the behavioral and memory effects of the peptide, while still needing further investigation to complete our knowledge, are relatively well understood. The structures involved are those innervated by extrahypothalamic vasopressinergic pathways (De Vries et al., 1985; Sofroniew, 1985) and the ones studied the most with respect to the effects of VP on learned behavior are limbic structures such as the septum (Dantzer et al., 1988; Engelmann et al., 1992; Everts and Koolhaas, 1997), the hippocampus (Kovacs et al., 1982, 1986; Metzger et al., 1993; Van Wimersma Greidanus and Maigret, 1996; Dietrich and Allen, 1997), and the amygdala (Roozendaal et al., 1992; Insel et al., 1993). Other structures less frequently studied but reported to also play a role in the behavioral effects of VP are the posterior thalamic area (including the parafascicular nucleus), the dorsal raphe nucleus, and the lateral habenula (Van

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Wimersma Greidanus et al., 1973, 1975). The behavioral and memory effects of VP in all these structures are not only mediated by vasopressin V_1 type receptors (De Wied et al., 1984; Ferris et al., 1988; Irvin et al., 1990; Dantzer and Bluthé, 1992; Willcox et al., 1992; Winslow et al., 1993; Alescio-Lautier et al., 1995), frequently detected in many brain structures (Ostrowski et al., 1992; Szot et al., 1994; Dubois-Dauphin et al., 1996), but also in some cases by oxytocin receptors (De Wied et al., 1991). More recently, the central involvement of the vasopressin V_2 type of receptor (De Wied et al., 1991; Popik et al., 1992) has been suggested but their cerebral location needs clarification.

This chapter focuses on the effects of VP on learning and memory in the hippocampus. It has been known for decades that the hippocampal system plays a prominent role in learning and memory (Eichenbaum et al., 1992; Jaffard and Meunier, 1993; Jarrard, 1995; Moser, 1995; Rolls, 1996) and that this structure contains endogenous VP. In rodents, from the extensive extrahypothalamic networks, the hippocampus receives only scattered vasopressinergic input in its ventral part. Indeed, VP immunoreactive fibers have been detected in the striata of the ventral hippocampal complex, including the paraventricular area, the claustrum, and the most ventral part of the dentate gyrus (De Vries et al., 1985; Castel and Morris, 1988). Most VP fibers in the ventral hippocampus originate in the medial amygdaloid nucleus (Sofroniew, 1985; Caffé et al., 1987). Unlike vasopressinergic innervation, both the dorsal and ventral parts of the hippocampus contain VP binding sites corresponding to the vasopressin V_{1a} receptor subtype. Some observations suggest that there may be vasopressin V_2 receptors on hippocampal neurons (Cheng and North, 1989; Hirasawa et al., 1994). Kato et al. (1995) state that expression of V_2 receptor mRNA in the hippocampus changes dynamically during the process of development. In addition to V_{1a} and perhaps V_2 vasopressin receptors, oxytocin receptors also appear to be present in the hippocampus (Freund-Mercier et al., 1987; Tribollet et al., 1988; Krémarick et al., 1993). Electrophysiological effects of VP have been reported in the hippocampus (Mühlethaler et al., 1983; Mizuno et al., 1984; Chepkova et al., 1995) and the dentate gyrus (Chen et al., 1993). These neurophysiological data indicate a relationship between VP and the hippocampus and support the findings on the behavioral effects of VP in this structure.

We shall first describe studies which have demonstrated the involvement of the hippocampus in the memory enhancing effect of VP and we shall point out the higher sensitivity of the ventral part of the structure to the behavioral action of VP. Next, we shall discuss the receptors involved in mediation of the behavioral effects of the peptide in the ventral hippocampus. Then we shall argue in favor of the fact that previous experience or the learning context before ventral hippocampal vasopressin treatment is an important factor in understanding the effects of VP on memory processes. Finally, we shall report data which provide support for the functional interaction of the vasopressinergic and noradrenergic systems in that structure.

The hippocampus: One of the central target structures for the memory enhancing effect of VP

The involvement of the hippocampus in the behavioral action of VP was first reported in studies using aversive conditioning. Small lesions of the dorsal hippocampus were found to modify the behavioral effects of peripherally administered VP on avoidance conditioning (Van Wimersma Greidanus and De Wied, 1976; Van Wimersma Greidanus et al., 1983a,b). In the hippocampus, a microinjection of 8 or 25 pg of VP improves retention of passive avoidance response, whether the injection is post-learning or pre-retention (Kovacs et al., 1986). In the dentate gyrus, a post-learning trial injection of VP improves passive avoidance behavior (Kovacs et al., 1979) and a pre-retention test injection of VP improves passive avoidance behavior of rats that have been made amnestic by pentylenetetrazol (Bohus et al., 1982). The relationship between the behavioral changes observed following the administration of VP and the behavioral consequences of the release of endogenous VP has been assessed by numerous authors (Van Wimersma Greidanus et al., 1979; Mens et al., 1982; Koob et al., 1985; Lebrun et al., 1987). Concerning the hippocampus, Laczi et al. (1983) detected a sharp

drop in VP during learning of avoidance conditioning when the level was measured just after the electric shock. This reduction was temporary. When the measure was taken after the retention session, a drop in the VP level was again observed eventhough the electric shock was not delivered in that condition, suggesting that hippocampal endogenous VP acts during retrieval processes. The involvement of hippocampal endogenous VP in aversive conditioning has also been assessed by the use of the anti-VP antibody. Indeed, post-learning injection of the anti-VP antibody (1/50th) into the dorsal hippocampus and the dentate gyrus produced a marked passive-avoidance retention deficit (Kovacs et al., 1982). Veldhuis et al. (1987) reported a similar result for the ventral hippocampus. Thus anti-VP in the hippocampus produces effects opposite to those obtained for VP, thereby confirming the role of this structure in the action of VP on the memory enhancement effect on aversive conditioning. Recently, Van Wimersma Greidanus and Maigret (1996) showed that endogenous VP in the hippocampus also plays a physiological role in social recognition.

Using discriminative learning in mice, we reported the involvement of the hippocampus in the enhancing effect of VP on memory retrieval and relearning when the peptide was injected intracerebroventricularly (i.c.v.) (Alescio-Lautier et al., 1987). The experimental task we used on the mice was a Go–No Go visual discrimination task similar to the one used for rats by Brito et al. (1982). The experimental device consists of two separate alleys, one white and one black. As the sessions progress, the animals must discriminate between the alley in which they are always reinforced and the alley in which they are never reinforced. The initial learning period consists of three daily sessions, each one including six Go trials and six No Go trials in random order. Performance is measured in terms of the animal's running time in each alley. Learning is manifested by a decrease in running time on Go trials and an increase in running time on No Go trials. The retention session takes place 24 days after the last learning session. This time interval was chosen following a preliminary study which allowed us to determine the forgetting curve of mice on this behavioral task (Alescio-Lautier and Soumireu-Mourat, 1986). The performance on the first Go trial and the first No Go trial is considered as reflecting the level of retrieval, and that obtained on the subsequent Go and No Go trials as reflecting the level of relearning. In this respect, the test series always began with a Go trial and a No Go trial or conversely. In this behavioral context, VP injected i.c.v. at a dose of 2 ng, 10 min before the retention session, improved retrieval and relearning performance, whereas control animals exhibited partial forgetting (Fig. 1A). In order to test whether or not the hippocampus is the central target for the memory enhancement effect of VP, we tested the effects of the peptide on animals with dorsal hippocampal lesions. Lesioning was done 2 days after initial learning and 2 ng of VP were injected i.c.v., as above. The results showed that hippocampal lesions reversed the effect of i.c.v. VP, while retrieval and relearning processes were not affected by the bilateral destruction of the dorsal hippocampus when the lesion was postponed after learning (Fig. 1B). Insofar as post-learning lesions of the dorsal hippocampus did not affect performance, the decline in performance cannot be ascribed to the lesion itself but to the combination of the lesion and the i.c.v. VP injection, since this structure seems to be essential for the expression of VP's behavioral effect. However, due to the non-specificity of the lesion, the same experiment was replicated but with immunoblockade of VP in the hippocampus (Alescio-Lautier et al., 1989). After having assessed the effects of the hippocampal VP immunoblockade itself, a 1/10 dilution of the antibody anti-VP (A-VP) into the dorsal hippocampus was injected before i.c.v. administration of the peptide. The results indicated that blocking endogenous VP in the dorsal hippocampus only slightly alters performance (Fig. 2A), whereas immunoneutralization followed by i.c.v. administration of VP inhibits the facilitating effect of the exogenous injection of the peptide (Fig. 2B). Thus, like the lesion of the hippocampus, which causes a deterioration in performance when it occurs in conjunction with i.c.v. addition of VP, VP immunoneutralization in this structure blocks the effects of the exogenous peptide. This gives us a more specific picture of the involvement of the hippocampus in the effects of VP administration.

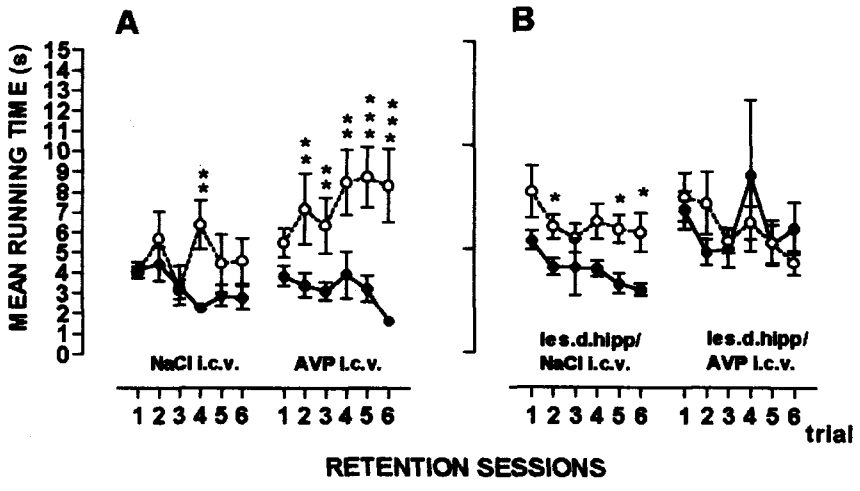


Fig. 1. Effects of intracerebroventricular (i.c.v.) injection of 2 ng of VP in dorsal hippocampal (d. Hipp)-lesioned mice on the retention of a discriminative learning task. Trial-by-trial analysis of mean (±SEM) Go (●) and No Go (○) running times during the retention session. (A) Effects of i.c.v. administered VP (VP i.c.v. group; $n = 9$) 10 min before the retention session and corresponding controls (NaCl i.c.v. group; $n = 9$). (B) Effects of i.c.v. VP on dorsal hippocampal lesioned mice (les.d.Hipp./VP i.c.v. group; $n = 11$) and corresponding controls (les.d.Hipp./NaCl i.c.v. group; $n = 9$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Greater sensitivity of the ventral part of the hippocampus to the memory enhancing effect of VP

Insofar as spontaneous forgetting can be alleviated following pre-test i.c.v. VP-treatment, and that this effect was blocked by immunoneutralization of the peptide in the dorsal hippocampus, we tested for the more direct involvement of this structure in the effect of VP by using in situ microinjection. Since Kovacs et al. (1986) reported more improvement of passive avoidance responses in rats when the peptide was injected into the ventral hippocampus, we compared the roles of the two hippocampal parts in the effect of VP on memory retrieval and relearning in mice. The role of both exogenous (Metzger et al., 1989) and endogenous (Alescio-Lautier et al., 1993) VP was assessed. To do so, the intrahippocampal administration of VP (25 pg) or of A-VP (1/10 dilution) was carried out 10 min before the retention session. The results showed that a bilateral injection of 25 pg of VP in the dorsal hippocampus improved retrieval performance without having a genuine effect on relearning, since the animals' performance on the last 5 Go and No-Go trials was no better than on the first Go and No Go trials (Fig. 3A). In contrast, a similar microinjection of VP in the ventral part of the hippocampus, which facilitated retrieval to the same extent as the dorsal treatment, led to a clear improvement in relearning (Fig. 3B). The immunoblockade of the endogenous peptide had a deleterious effect on performance, while the analogous administration of exogenous VP led to facilitation. In other words, memory retrieval deteriorates substantially regardless of where in the hippocampus the VP immunoblockade occurs, whereas the presence or absence of relearning is a function of the location of the treatment in the hippocampus: animals treated in the dorsal hippocampus are capable of relearning the task (Fig. 2A), while ventrally treated ones are not (Fig. 3C).

In order to assess the existence of potentially non-specific effects of VP or its immunoblockade, we tested for the impact of a microinjection of VP or A-VP on a locomotor task performed in the apparatus used for the visual discrimination task. This also allowed us to compare the animal's behavior in a non-associative context. The results showed that treatments delivered in the dorsal hippocampus

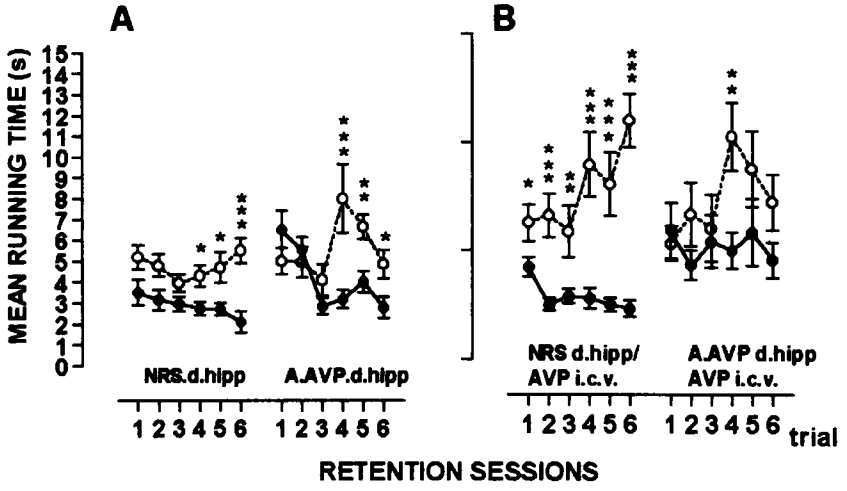


Fig. 2. Effects of VP antibody (A-VP) injected into the dorsal hippocampus (d.hipp) followed by i.c.v. VP (2 ng) on the retention of a discriminative learning task. Trial-by-trial analysis of mean (±SEM) Go (●) and No Go (○) running times during the retention session. (A) Effects of A-VP (1/10e dilution) injected into the dorsal hippocampus (A-VP d.hipp group; $n = 8$) and corresponding controls: mice injected with normal rabbit serum (NRS, 1/10e dilution) (NRS d.hipp group, $n = 9$). (B) Effects of A-VP injected into the dorsal hippocampus followed by i.c.v. VP (A-VP d.hipp/VP i.c.v./; $n = 11$) and corresponding controls (NRS d.hipp/VP i.c.v.; $n = 10$). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

affect locomotor activity, while the same treatments in the ventral hippocampus affect only performance linked to the non-associative context (Metzger et al., 1993). These results and their correlation with those obtained on the visual discrimination task led us to hypothesize that the involvement of VP in the

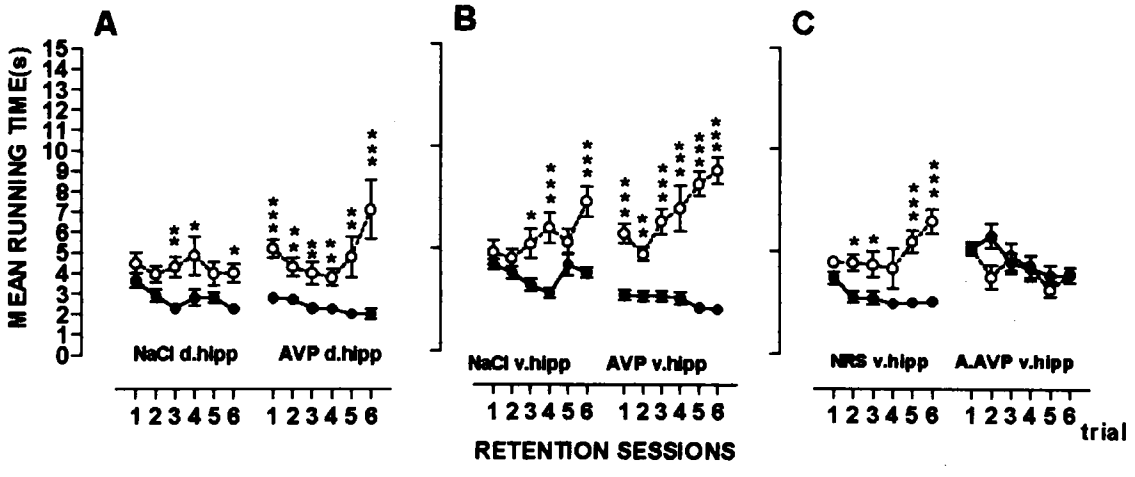


Fig. 3. Involvement of the hippocampus in the effect of VP on the retention of the visual discrimination task. Trial-by-trial analysis of mean (±SEM) Go (●) and No Go (○) running times during the retention session. (A) Effects of VP (50 pg per animal) in the dorsal hippocampus (VP d.hipp group; $n = 10$) and corresponding controls (NaCl d.hipp group; $n = 10$). (B) Effects of VP in the ventral hippocampus (VP v.hipp group; $n = 10$) and corresponding controls (NaCl v.hipp group; $n = 10$). (C) Effects of A-VP (1/10e dilution) in the ventral hippocampus (A-VP v.hipp group; $n = 10$) and corresponding controls (NRS v.hipp group; $n = 9$). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

dorsal hippocampus might be linked to mechanisms affecting the subject's emotional and arousal states, whereas the VP effect in the ventral hippocampus might be linked to mechanisms that are more specifically involved in retrieval and relearning, since animals ventrally treated with VP adapt their behavior to the experimental context. The fact that immunoblockade of VP in the dorsal hippocampus can reduce retrieval without affecting relearning (Fig. 2A) is surprising and raises the question of the nature of the link between these two processes. Their dissociation could be explained by the fact that because the dorsal hippocampal VP is involved in the subject's emotional state, its blockade impairs retrieval performance; as the causes of this impairment may be non-specific, the animals may be able to relearn the task. Some results that are in line with the involvement of dorsal hippocampal VP in retrieval processes were reported by Laczi et al. (1983), which demonstrated changes in VP content in that structure and in the cerebrospinal fluid after electric footshock during passive avoidance behavior. However, the same authors also reported a change in VP contents in the hippocampus after the retention session of passive avoidance behavior. Since the electric shock was not delivered during retention, changes in VP concentration could be ascribed to the association between the memory of the electric shock and the environment. In other words, hippocampal endogenous VP may act during retrieval processes. If so, the impairment of retrieval processes could correspond to the real involvement of dorsal hippocampal VP in retrieval processes. Indeed, previous studies have reported a reduction in avoidance scores during retention of passive avoidance behavior after the microinjection of 1/50 diluted A-VP serum into the dorsal hippocampus (Kovacs et al., 1982; Van Wimersma Greidanus and Veldhuis, 1985; Veldhuis et al., 1987).

All things considered, it seems beyond all doubt that exogenous and endogenous VP in the dorsal hippocampus are involved in memory retrieval. The specificity of this involvement remains to be demonstrated, however. In contrast, the involvement of VP in the ventral hippocampus seems more specific, since results of a previous study (Metzger et al., 1993) suggested that the deleterious effect induced by the immunoneutralization of endogenous VP in the ventral hippocampus reflects the inability of animals to remember the reinforcement-color association, but not the reinforcement itself. Thus, the impairment observed when ventral hippocampal endogenous VP was blocked should be due to a deterioration in the associative function. For this reason, and also because VP in this part of the hippocampus seems clearly to be involved in both retrieval and relearning, studying the ventral part of the structure in further research appeared to be a good means of gaining insight into the effects of vasopressin on these processes.

VP enhancing effect in the ventral hippocampus is mediated through V_1 and oxytocin receptors

In order to test for the involvement of V_1, V_2, and oxytocin receptors in the effects of VP on memory retrieval and relearning, we performed a microinjection into the ventral hippocampus of the vasopressin V_1 receptor antagonist, $d(CH_2)_5Tyr(Me)VP$, the vasopressin V_2 receptor antagonist $d(CH_2)_5$-D-Ile^2,Ile^4, Arg^8]-vasopressin, or the oxytocin receptor antagonist, [$d(CH_2)_5$,$Tyr(Me)^2$,Thr^4,$Tyr\text{-}NH_2^9$]-OVT prior to the microinjection of VP. Before doing so, the dose at which the antagonist when administered before the retention session would not affect retrieval and relearning had to be determined. This measure would enable us later to determine whether or not the effects of VP persisted after the administration of the antagonist. Considering the substantial involvement reported in the literature of V_1 type receptors in the central behavioral effect of VP, we determined the dose with the V_1 receptor antagonist. A 1-ng bilateral dose of the V_1 receptor antagonist resulted in a deterioration of retrieval and relearning. Due to these deleterious effects, this dose was not used to test the effects of the antagonist on VP facilitation. However, an interesting point concerning this dose is that the magnitude of the observed negative effect was found to be comparable to that previously observed following immunoblockade of endogenous VP. This confirms the involvement of endogenous VP in retrieval and relearning. When the antagonist produced no effect on the performance, the dose was 50 pg per animal (Alescio-Lautier et al., 1995). It is also interesting to note that this was

the dose for which VP was found to be behaviorally active when injected bilaterally into the hippocampus. We tested this dose for the other antagonists and found that they also had no effect on performance.

When the administration of VP was preceded by the administration of one of the three antagonists, the facilitating effect was antagonized by the V_1 and oxytocin receptor antagonists but not by the V_2 receptor antagonist (Fig. 4). The blockade of the facilitating effect of VP by the V_1 receptor antagonist (Fig. 4A) is consistent with results in the literature showing in other brain sites and for other behaviors that the effects of VP are mediated by vasopressin V_1 receptors (De Wied et al., 1984, 1991; Ferris et al., 1988; Irvin et al., 1990; Dantzer and Bluthé, 1992; Willcox et al., 1992; Winslow et al., 1993). We have shown that in the ventral hippocampus, oxytocin receptors are also involved in the enhancing effect of VP on retention performance (Fig. 4B). The involvement of these receptors in the enhancing effect of the peptide was also reported by De Wied et al. (1991) for memory consolidation and by Roozendaal et al. (1992, 1993) for other functions of the peptide. In contrast, Popik et al. (1992) reported that the effect of vasopressin on social recognition in rats was not affected by a previous injection of an oxytocin receptor antagonist into the lateral septum. As a general rule, though, in structures such as the ventral hippocampus and amygdala, which contain both vasopressin V_1 and oxytocin receptors (Krémarick et al., 1993), the effect of VP on memory processes depends not only on the activation of their own V_1 receptors (Winslow and Insel, 1993; Alescio-Lautier et al., 1995) but also on that of oxytocin receptors (De Wied et al., 1991; Roozendaal et al., 1992, 1993). The results obtained by Van Wimersma Greidanus and Maigret (1996), which showed that the presence or local release of VP and oxytocin in the ventral hippocampus are of physiological importance for social recognition, corroborate the idea that in the ventral hippocampus, a part of VP's effects might have an oxytocinergic component.

The lack of involvement of vasopressin V_2 receptors in retrieval and relearning (Fig. 4C) is inconsistent with the results reported by De Wied et al. (1991) and Popik et al. (1992), who found, respectively, that an i.c.v. or an intra-septal injection of a vasopressin V_2 receptor antagonist before the injection of VP blocks the enhancing effect of vasopressin on the retention of a passive avoidance task or on social recognition. However, these studies differ from our own on several points. First, they assessed the effect of the peptide on memory consolidation, whereas we tested its effect on retrieval processes. Second, the site of injection of the two peptides differed across studies, since De Wied et al. (1991) injected into the lateral ventricle and Popik et al. (1992) into the lateral septum. Thus, another explanation of the discrepant results (which does not rule out the one presented above) would be that the ventral hippocampus does not contain vasopressin V_2 receptors. Indeed, although they have been detected in the central nervous system (Ostrowski et al., 1992), their presence in the hippocampus is still under debate. Hirasawa et al. (1994) demonstrated the existence of vasopressin V_2 receptor mRNA in the hippocampus whereas Kato et al. (1995) reported that their expression in the hippocampus changes dynamically during the process of development in rats, since it was found to decrease with age in newborns and could not be detected in rats more than 2 weeks old. Considering the location of vasopressin V_2 receptors, the results reported by Popik et al. (1992) are surprising, since these receptors were not detected in the lateral septum. For these authors, one explanation is that the action of vasopressin on social recognition is mediated by a vasopressinergic receptor that does not discriminate beween V_1 and V_2 antagonists, which are peptides developed to block the peripheral rather than central effects of vasopressin.

In addition, our results suggest that the effect of VP on retrieval and relearning processes is mediated by both vasopressin V_1 and oxytocin receptors. The involvement of two receptor types on these processes may appear redundant. In this light, the blocking of only one receptor type could not prevent the enhancing effect of VP. On the contrary, we showed that vasopressin V_1 receptors do not supplant oxytocin receptors in the beneficial effect of VP, and vice versa. This suggests the existence of an as yet-to-be-determined link between vasopressin V_1 and oxytocin receptors. Another

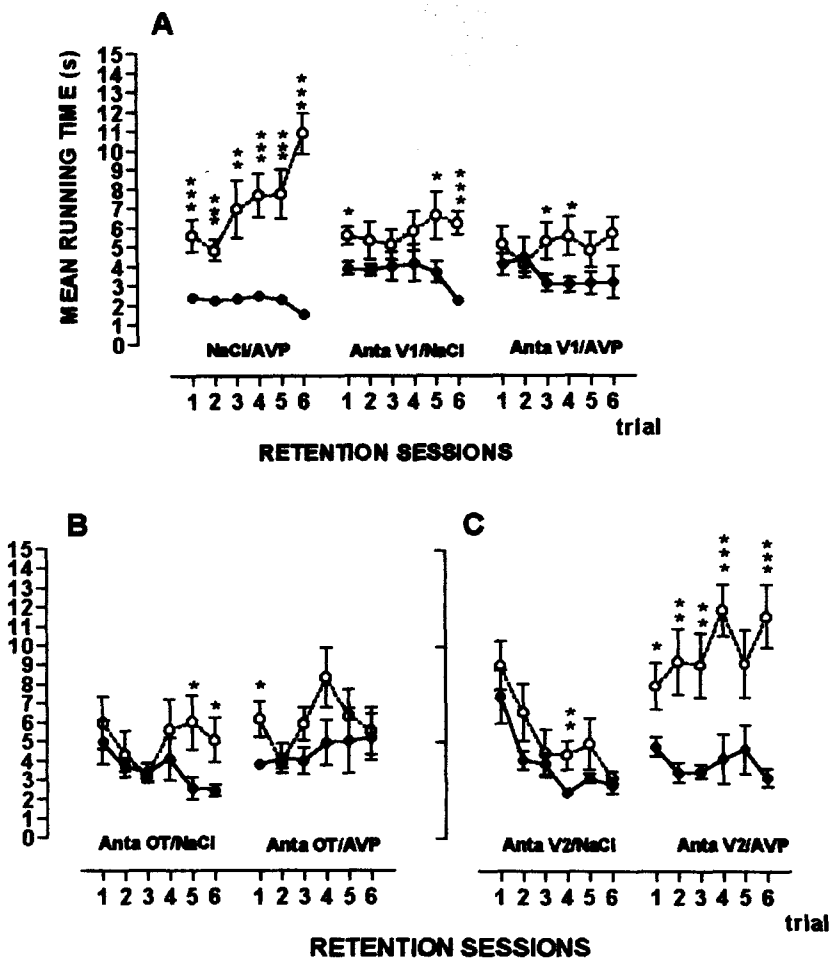


Fig. 4. The memory enhancing effect of VP in the ventral hippocampus is antagonized by V_1 and oxytocin receptor antagonists but not by the vasopressin V2 receptor antagonist. Trial-by-trial analysis of mean (±SEM) Go (●) and No Go (○) running times during the retention session of the visual discrimination task. (A) Effects of bilateral microinjection of NaCl (0.3 µl volume) followed by VP (25 pg, NaCl/VP group; $n = 10$) or $d(CH_2)_5Tyr(Me)VP$, V_1 receptor antagonist (25 pg) followed by NaCl (Anta V_1/NaCl group; $n = 10$) or $d(CH_2)_5Tyr(Me)VP$ (25 pg) followed by VP (25 pg, anta V_1/VP group; $n = 10$). Injections of vehicle or peptides were performed 15 min prior to the retention session. The two injections were separated by a 5-min interval. (B) Effects of bilateral microinjection of $[d(CH_2)_5,Tyr(Me)^2,Thr^4,Tyr\text{-}NH_2^9]$-OVT, oxytocin receptor antagonist (25 pg), followed by NaCl (anta OT/NaCl group; $n = 10$) or $[d(CH_2)_5,Tyr(Me)^2,Thr^4,Tyr\text{-}NH_2^9]$-OVT (25 pg) followed by VP (25 pg, anta OT/VP group; $n = 10$). (C) Effects of bilateral microinjection of $[d(CH_2)_5\text{-D-Ile}^2,Ile^4,Arg^8]$-vasopressin, V_2 receptor antagonist (25 pg), followed by NaCl (anta V_2/NaCl group; $n = 10$) or $[d(CH_2)_5\text{-D-Ile}^2,Ile^4,Arg]$-vasopressin (25 pg) followed by VP (25 pg, Anta V_2/VP group; $n = 10$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

explanation would be the one hypothesized by De Wied et al. (1991), that only one receptor type is involved in the effect of the peptide. This receptor would have the same affinity for both vasopressin V_1 and V_2 receptor antagonists and the oxytocin receptor antagonist. Thus, we still need to determine what receptors in the ventral hippocampus are involved in the enhancing vasopressin memory effect. Further studies regarding the molecular characterization of the receptors sensitive to vasopres-

sin in the ventral hippocampus would be useful in resolving this issue.

Mnemonic context is an important factor in understanding the effect of VP on memory processes in the ventral hippocampus

For mice learning the visual discrimination task, we previously reported that the subcutaneous administration of VP either reduced or improved performance, depending on whether the treatment was performed at the beginning or in the middle of the learning processes (Alescio-Lautier and Soumireu-Mourat, 1990). Except for retrieval processes, where most of the published studies using pre- or post-learning treatment assign VP an enhancing effect, the literature reports both facilitatory effects (Bohus et al., 1978; Buresova and Skopkova, 1982; Davis et al., 1982; Dantzer et al., 1988; De Wied et al., 1991) and deleterious effects (Hostetter et al., 1980; Sahgal et al., 1982; Van Haaren et al., 1986; Alexinsky and Alliot, 1987; Engelmann et al., 1992; Ebenezer, 1993). This inconsistent effect of VP on acquisition and memory processes may be due to the fact that the treatment was never applied at the same point in the learning process. With avoidance conditioning, Rigter (1982) suggested that differences in the degree to which animals were familiarized with the training situation prior to acquisition accounted for the discrepancy between the results of various studies. Indeed, this author reported that VP may or may not facilitate memory consolidation, depending on the nature of the animal's prior experience with the training situation. In the light of these findings, we examined the effects on behavior of VP applied to the ventral hippocampus, in relation to the time of treatment during learning (Paban et al., 1996). A visual discrimination task was used and VP was bilaterally microinjected at a dose of 25 pg per animal in the ventral hippocampus before either the first or the second learning session, or immediately after either the first or the second learning session.

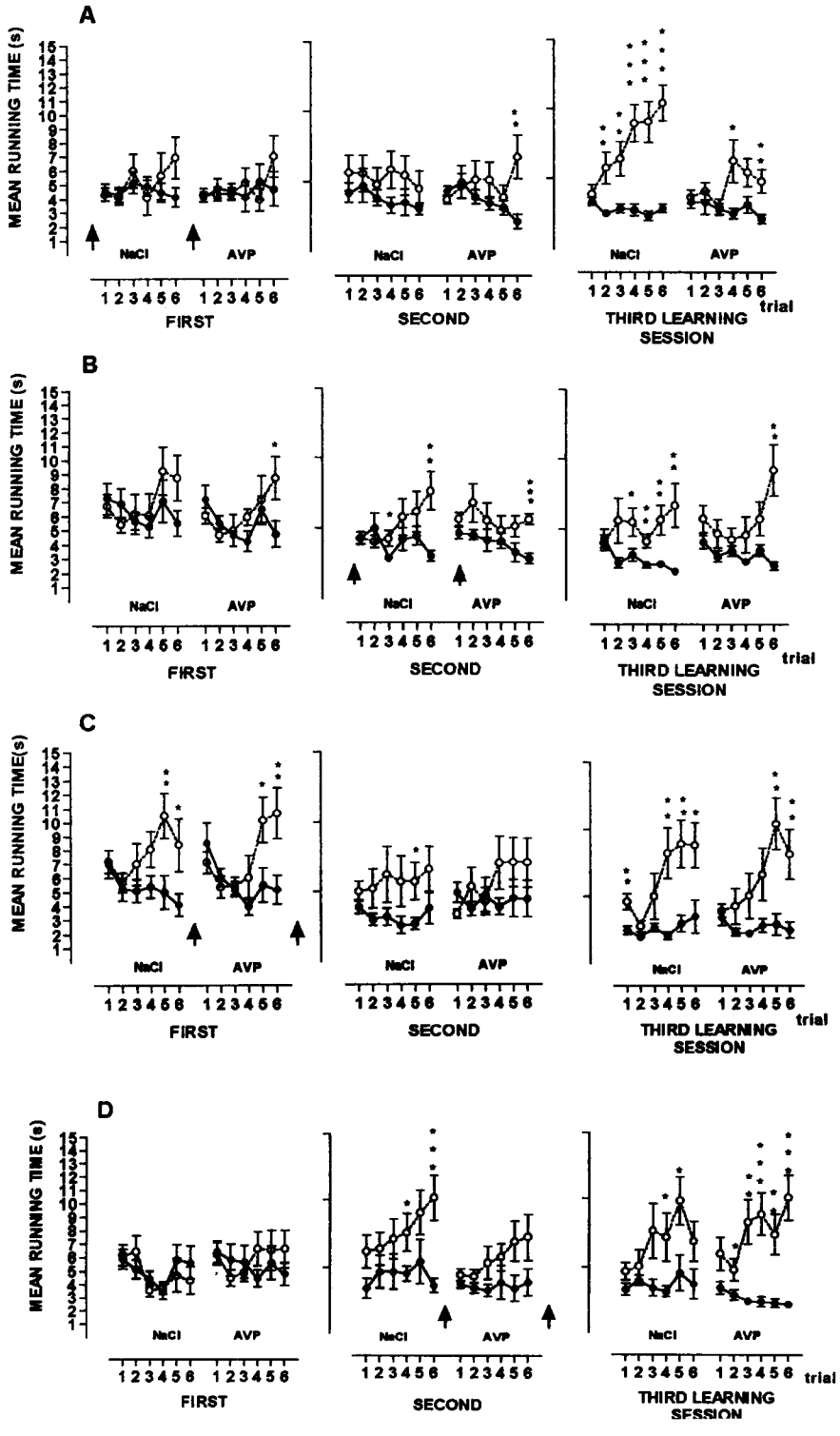
For pre-session treatment, we showed that VP injected before the first or second learning session delayed learning or induced no effect on learning, respectively (Fig. 5A,B). Delayed learning after pre-first session VP treatment has been clearly demonstrated on the same behavioral task following peripheral (Alescio-Lautier and Soumireu-Mourat, 1990) and i.c.v. (unpublished data) VP treatment. In this situation, the inability of the treated animals to learn the task suggests that a deleterious effect of VP appears when the animals have no information about the task to learn. The fact that VP did not generate a deleterious effect when it was injected before the second learning session is consistent with this assumption. Moreover, the VP did not generate the same behavioral effect when the treatment was done prior to the first or second learning sessions, suggesting that the effect of the peptide depends on the animal's past experience in the same learning context.

In our behavioral procedure, which used several daily learning sessions each composed of several trials, it is difficult to distinguish between learning and retrieval processes. A case in point is when the treatment was given before the second learning session, which can also be regarded as a retention session occurring one day after learning. We therefore tested the effect of VP on learning in this situation, i.e. across the six Go or No Go trials, but also on retrieval, i.e. on the first Go trial and on the first No Go trial. We showed that in this condition, the peptide had no effect on performance. This result may seem inconsistent with numerous findings in the literature, particularly concerning aversive conditioning, where an enhancing effect of VP on retention is reported, unless we take into account the fact that, unlike in passive avoidance conditioning, one learning session is not sufficient for learning the visual discrimination task and the animals' performance at the time of the second learning session is unreliable. Thus, in the case of poor learning, intrahippocampal VP treatment does not affect both the retrieval processes and the animal's capacity to learn. In contrast, for animals who have learned the task well, at a time when control animals displayed forgetting, i.e. 24 days after learning, we showed that intrahippocampal VP treatment before the retention session improved retrieval and relearning (Fig. 3B). Regarding retrieval, it seems that the involvement of the peptide depends on the quality of the previously stored information. At the end of the first learning session,

poor performance was obtained, and on the next day when VP or NaCl treatment was administered, both treated and control animals displayed no learning on the first Go and No Go trials (Fig. 5B). However, when the animals were allowed to perform three learning sessions, their performance was good, and when their memory was tested 24 days later, the control animals displayed substantial forgetting on the first Go and No Go running times, whereas VP treated animals improved considerably, indicating that the peptide increased the accessibility of information in long-term memory (Fig. 3B). Considering the animals' capacity to learn (second learning session, Fig. 5B) or relearn (retention session, Fig. 3B) the task, it appears that the effectiveness of the peptide depends on the level of learning at the time of the treatment. In other words, a positive effect of VP is obtained if the animals have already detected the cues needed for proper learning. Indeed, 24 days after learning, VP treated animals displayed good retrieval, which points out the high quality of the memory trace that allowed them to process the current information better than control animals, and thus to display better relearning. In contrast, when the peptide was injected before the second learning session, the animals displayed poor performance, indicating a memory trace of poor quality and consequently, little acquired knowledge about the task to be learned. Under these conditions, the peptide had no effect.

Surprisingly, some authors have not considered the animal's past experience as a factor which could influence the behavioral effect of VP, and consequently, they have not taken this factor into account in the analysis of VP's effect. Thus, it is difficult for the reader to form an opinion on this issue, because the animal's prior experience at the time of treatment has not always been described by the experimenter. This is often the case for the habituation and pre-training periods. Baranovska et al. (1983) reported no VP effect on the learning of an avoidance conditioning task when the peptide was administered 15 min before the first learning session. In other studies, the effect of VP has been evaluated on learning using pre-session administration, but when the animal has already performed either a pre-training session or one learning session. Sara et al. (1982) reported improved learning in a food-rewarded brightness discrimination task when the peptide LVP was subcutaneously injected prior to the learning session. This session was preceded by both an adaptation period and a training session of 40 trials, so in this case, the animals knew the experimental context prior to learning. In a social learning paradigm, Siegfried et al. (1984) reported that learned submissive behavior was influenced differently by subcutaneously administered vasopressin, depending on the time of application. Pre-trial treatment induced an amnesic effect, whereas a facilitatory effect was obtained by pre-retention treatment. It has also been shown that VP treatment induces a different effect according to whether it is applied before learning or before extinction of avoidance conditioning. Koob et al. (1981) showed that subcutaneous administration of VP resulted in a greater effect on extinction than on acquisition in rats. Hamburger et al. (1985) reported VP response differences in a mice strain, and differences linked to the acquisition or extinction situation in a two-way shuttle box. The treatment was performed one hour before each session. Under these conditions, for BALB/c mice (which was the strain used in our study), VP inhibited acquisition and generated few effects on extinction. All of these findings are in line with the fact

Fig. 5. Effects of ventral-intra-hippocampal injection of VP at different times in the learning process. Trial-by-trial analysis of mean (±SEM) Go (●) and No Go (○) running times during the learning sessions of the visual discrimination task. (A) Effects of bilateral injection of VP (25 pg) 10 min before the first learning session (VP group; $n = 10$) and corresponding controls (NaCl group; $n = 10$). (B) Effects of bilateral injection of VP (25 pg) 10 min before the second learning session (VP group; $n = 10$) and corresponding controls (NaCl group; $n = 10$). (C) Effects of bilateral injection of VP (25 pg) 10 min after the first learning session (VP group; $n = 10$) and corresponding controls (NaCl group; $n = 10$). (D) Effects of bilateral injection of VP (25 pg) 10 min after the second learning session (VP group; $n = 10$) and corresponding controls (NaCl group; $n = 10$). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.


A
MEAN RUNNING TIME (s)
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1 2 3 4 5 6 1 2 3 4 5 6
FIRST
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B
C
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that when vasopressin treatment is done at an early stage in the learning process, a deleterious effect or no effect is obtained. Since, this effect occurs at every site of injection, i.e. subcutaneous, i.c.v., and in situ, it seems specific to VP and not to a particular brain area such as the ventral hippocampus, as our results might suggest.

As with pre-session injections, we showed that the post-session injection of VP induces different effects, depending on the time of treatment. When injected after the first learning session, the peptide slightly delayed performance (Fig. 5C), whereas when the injection took place after the second learning session, it enhanced learning (Fig. 5D). Thus, it seems that the effects of VP on memory consolidation may depend (as on learning and retrieval processes) upon the previous level of performance acquired by the animals. Rigter (1982) has already demonstrated the role of prior experience in the effect of the post-treatment of VP. This author used a step-through passive avoidance task to test various behavioral variables for the efficacy of the post-trial injection of VP, and showed that in the absence of pre-training or of any one of the pre-training components, the post-trial enhancement effect of VP was lost. The author concluded that 'VP may facilitate memory consolidation in rats depending on the nature of prior experience with the training situation'. This could explain the discrepancy between some of the results, especially those reported by De Wied et al. for passive avoidance conditioning (De Wied et al., 1976, 1991; Bohus et al., 1978) versus those reported by Hostetter et al. (1980) and Sahgal et al. (1982) on the same behavioral task. This is consistent with our own results which showed that on the same behavioral task and in the same treatment situation (post-trial), the effect of VP varied with the information available at the time of treatment. In other words, VP only facilitates memory consolidation when the animal is able to extract relevant stimuli for proper learning. Strupp (1989) reported that the subcutaneous, post-trial administration of VP4-9 alleviated the forgetting exhibited by control animals in a radial maze. However, a relation was observed between the proficiency of the rats and the degree to which their performance was improved by post-trial VP treatment, suggesting to this author that physiological differences between animals varying in mnemonic ability may account for their different responses to peptide treatment. For extinction behavior, the effect of VP was difficult to interpret due to the behavioral processes involved in the extinction, which are not entirely clear. Using an eight-arm radial maze, Packard and Ettenberg (1985) showed that subcutaneous VP injection after the first extinction trial potentiated radial maze extinction behavior. This result suggests that VP improved the learning that occurred during extinction. In a positively reinforced autoshaped lever-touch response, i.c.v. administration of VP after the last day of training was found not to alter responses on consecutive extinction sessions (Car and Murtazina, 1994). In social behavior, i.c.v. or intraseptal administration of VP in a post-trial paradigm was found to enhance social recognition in rats (Le Moal et al., 1987; Dantzer et al., 1988). Looking at all of the reported data, the effects of VP seem unclear. In our mind, this cannot be ascribed to discrepant results, because in most cases VP effects were tested under different conditions. This may be precisely why the effects of the peptide vary.

Possible functional interaction of the vasopressinergic and noradrenergic systems in the ventral hippocampus

In that study, we attempted to determine the implication of hippocampal adrenergic receptors in the enhancing effects of VP on retrieval and relearning of the Go and No Go appetitive discrimination tasks. The data that gave rise to this work was first obtained by Kovacs et al. (1979) and more recently, by Brinton (1990). Kovacs et al. (1979) showed that the memory-enhancing effects of vasopressin were blocked by lesions of the dorsal noradrenergic bundle, which is the source of hippocampal noradrenergic innervation. These authors suggested that vasopressinergic effects were dependent upon the noradrenergic innervation of the hippocampus. Biochemical data showed that vasopressin acts to potentiate the accumulation of noradrenaline-induced cyclic adenosine monophosphate (cAMP) in the hippocampus. The action of the peptide is conditionally dependent upon the

activation of beta-adrenergic receptors (Brinton, 1990). Since both types of alpha- and beta-adrenergic receptors have been found in the hippocampus (Young and Kuhar, 1980; Jones et al., 1985), we tested the involvement of each type of adrenergic receptor in the behavioral effect of VP.

Role of alpha-adrenergic receptors in the enhancing effect of VP on retention performance

To examine the role of alpha-adrenergic receptors in the enhancing VP effect on memory retrieval, we used phentolamine, an antagonist which equally antagonizes alpha1- and alpha2-receptor subtypes. Thus, we first defined the effect of phentolamine alone on the retention performance of the visual discrimination task using a pre-retention injection of the antagonist in the ventral hippocampus at a dose of 2 μg per animal. Then, the effect of pre-treatment by phentolamine on the effect of VP treatment by phentolamine followed by VP was tested (Metzger et al., 1994).

Phentolamine injected 10 min after the retention session improved retrieval and slightly affected relearning (Fig. 6A). Sara and Devauges (1989) observed an increase in retrieval after treatment by an alpha2-adrenergic receptor antagonist, idazoxan. In the cerebral cortex and hippocampus, the noradrenaline release was found to decrease after the stimulation of presynaptic alpha2 receptors (Jackisch et al., 1985). These receptors are classically described as inhibitory autoreceptors which regulate noradrenaline release (Aghajanian et al., 1977). Thus, the effects of phentolamine on retrieval processes may correspond to the blockade of alpha2 receptors, which in turn triggers an increase in the quantity of available noradrenaline in the ventral hippocampus. In other words, the improvement observed after phentolamine administration could result from the lack of inhibitory regulation of the noradrenaline release.

When phentolamine was injected prior to VP, the effect of the peptide persisted even when the alpha-adrenergic receptors were blocked (Fig. 6B). Furthermore, we found an improvement in global performance after administration of phentolamine followed by VP as compared to the performance triggered after administration of plus saline VP, suggesting the combined action of the alpha-adrenergic and vasopressinergic receptors. Devauges and Sara (1991) showed that stimulation of the locus coeruleus, which led to an increase in noradrenaline release in the forebrain, resulted in an improvement in retrieval. This effect was blocked

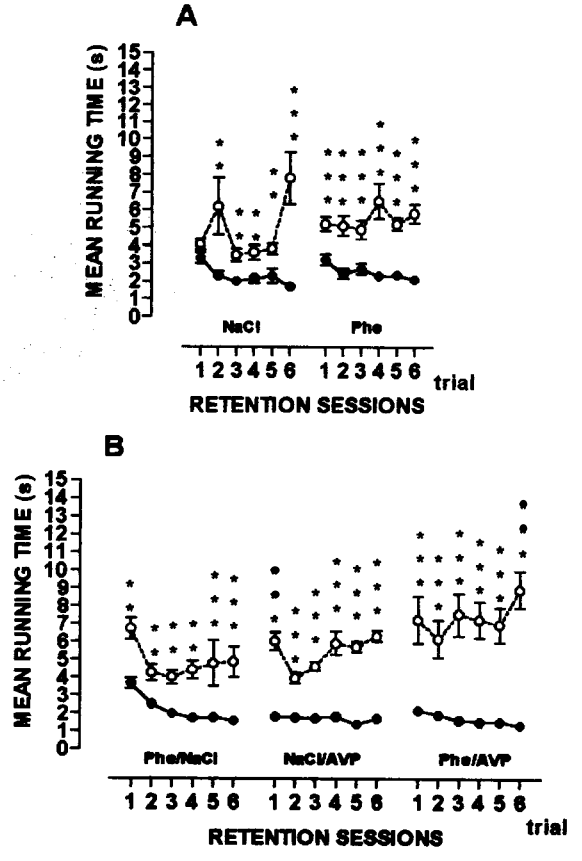

Fig. 6. Effects of pre-treatment by phentolamine on the enhancing VP effects on retention of a visual discrimination task. Trial-by-trial analysis of mean (±SEM) Go (●) and No Go (○) running times during the retention session. (A) Effects of phentolamine, antagonist of alpha 1- and alpha 2-adrenergic receptors, bilaterally administered in the ventral hippocampus (1 μg/animal) 10 min prior to the retention session (phe group; $n = 10$) and corresponding controls (NaCl group; $n = 10$). (B) Effects of bilaterally administered phentolamine (1 μg) in the ventral hippocampus followed by VP (25 pg, Phe/VP group; $n = 9$) and corresponding control groups: phentolamine followed by NaCl (phe/NaCl group; $n = 9$) or NaCl followed by VP (NaCl/VP group; $n = 9$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

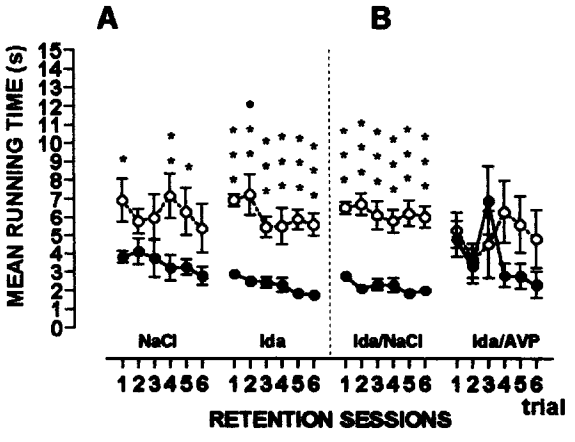

Fig. 7. Effects of pre-treatment by idazoxan on the enhancing VP effects on retention performance of a visual discrimination task. Trial-by-trial analysis of mean (±SEM) Go (●) and No Go (○) running times during the retention session. (A) Effects of idazoxan, antagonist of alpha 2-adrenergic receptors, bilaterally administered in the ventral hippocampus (0.63 μg/animal), 10 min prior to the retention session (Ida group; $n = 10$) and corresponding control (NaCl group; $n = 10$). (B) Effects of bilaterally administered idazoxan (0.63 μg) in the ventral hippocampus followed by VP (25 pg, Ida/VP group; $n = 10$) and corresponding control group: idazoxan followed by NaCl (Ida/NaCl group; $n = 10$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

by the intraperitoneal administration of propranolol, antagonist of the beta-adrenergic receptors. Thus, the improvement in retrieval might have been achieved by stimulation of the beta-adrenergic receptors. Moreover, it has been suggested that the beta-adrenergic and vasopressinergic receptors are linked to each other (Brinton, 1990). Given these facts, in conjunction with the hypothesized primary implication of the alpha2-adrenergic receptors through the regulation of available noradrenaline, we could interpret the effect of the administration of phentolamine followed by VP as the consequence of the induction of an increase in the co-stimulation of beta-adrenergic and vasopressinergic receptors by endogenous noradrenaline. In this interpretation, the alpha2 receptors may be involved as regulators and the beta receptors as effectors. However, an additional study did not confirm this assumption. Indeed, although administration of idazoxan, an alpha2 receptor antagonist, prior to the retention test markedly improved performance (Fig. 7A), it induced a deleterious effect when given prior to the VP treatment (Fig. 7B). This double treatment differed from that of phentolamine prior to VP by its action on alpha1-adrenergic receptors; following phentolamine, alpha1 receptors were inhibited, whereas following idazoxan, these receptors were either quiet or activated by endogenous noradrenaline. Since it is likely that the two treatments had a similar action on beta-adrenergic receptors, their activation cannot be responsible for the increase of the enhancing effect of VP by phentolamine as we previously thought. This increase may instead be induced by the inhibitory action of phentolamine on alpha1-receptors. Moreover, the fact that idazoxan and VP improved performance when given separately while decreasing it when injected together, suggests that the relationship between VP receptors and alpha-adrenergic receptors may be interactive rather than additive. Concerning VP receptors, we reported earlier in this chapter that it was not only V_1 type receptors that were responsible for the enhancing VP memory retrieval effect, but also oxytocin receptors. It would be worthwhile to consider this in our next studies.

Role of beta-adrenergic receptors in the enhancing effect of VP on retention performance

Using propranolol, an antagonist of beta-adrenergic receptors, to test for the possible involvement of these receptors in the effect of VP, we showed that the administration of this antagonist at a dose of 2 μg per animal has no influence on retrieval and relearning (Fig. 8A). While it is known that treatment by propranolol usually produces a decrease in performance when administered during the training sessions (Staneva-Stoytcheva et al., 1989; Decker et al., 1990), its effects on consolidation and retention are much less well understood (Gallagher et al., 1977; Sternberg et al., 1985; McGaugh, 1989). When we pre-treated with propranolol, the effect of VP on both retrieval and relearning was blocked (Fig. 8B). Thus, the memory enhancing effect of VP seems to depend on the functional state of the beta-adrenergic receptors. Studies by Kovacs et al. (1979) showing that lesions of the dorsal noradre-

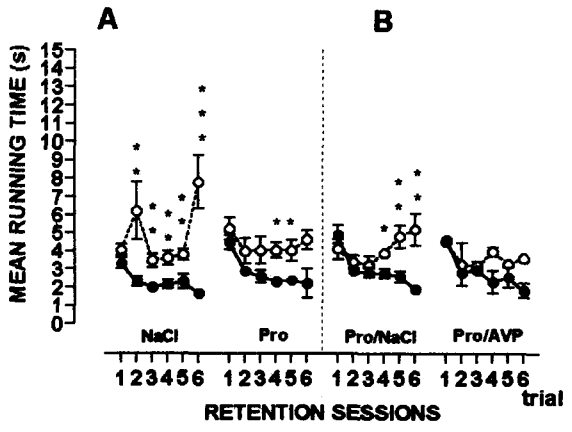

Fig. 8. Effects of pre-treatment by propranolol on the enhancing VP effects on retention of a visual discrimination task. Trial-by-trial analysis of mean (±SEM) Go (●) and No Go (○) running times during the retention session. (A) Effects of propranolol, antagonist of beta-adrenergic receptors, bilaterally administered in the ventral hippocampus (1 μg/animal), 10 min prior to the retention session (Pro group; $n = 10$) and corresponding controls (NaCl group; $n = 10$). (B) Effects of bilaterally administered propranolol (1 μg) in the ventral hippocampus followed by VP (25 pg, Pro/VP group; $n = 9$) and corresponding control group: propranolol followed by NaCl (Pro/NaCl group; $n = 9$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

nergic bundle block the effect on retention of the intrahippocampal administration of VP are consistent with our data and suggest the implication of the noradrenergic innervation in the effect of the peptide in the hippocampus. Moreover, our results corroborate the biochemical data obtained by Brinton and McEwen (1989) who used an in vitro technique to demonstrate the specific implication of the beta-adrenergic receptors in the effects of VP on cAMP accumulation in the hippocampus. In the present study, the inactivation of beta-adrenergic receptors blocked the effects of VP but the mice receiving these two treatments and the mice receiving either propranolol alone or propranolol plus saline, even though their performance decreased slightly, could relearn the task. While the propranolol dose effect was unknown in our conditions, the dose used in this intrastructure administration was high. Thus, we can argue that the complete blockade of beta-adrenergic receptors occurred after propranolol treatment. We reported above that either the immunoneutralization of endogenous VP in the ventral hippocampus (Fig. 3C) or the administration of an antagonist of vasopressinergic type 1 receptors $d(CH_2)_5Tyr(Me)VP$ (Fig. 4A) resulted in the complete deterioration of both retrieval and relearning. Since we did not find the total deterioration of retrieval and relearning after propranolol treatment as we did in $d(CH_2)_5Tyr(Me)VP$ treatment, we suggest that the peptide may act by some other mechanism than the one involving the beta-adrenergic receptors, at least as far as relearning processes are concerned. The blockade of the enhancing effect of VP by idazoxan supports this assumption. However, further studies will be necessary to clarify the role played by each receptor type or subtype in retrieval and relearning processes, and to determine the relationships that might exist between them.

Conclusion

All reported data on the role played by VP in the hippocampus in memory processes suggest that VP does not affect information intake, but may contribute to its processing, storage, and retrieval. However, the involvement of VP in memory consolidation and retrieval is significant when the animal has already extracted the stimuli needed for proper learning; otherwise, VP delays acquisition. This means that the peptide contributes to information storage whenever that information has acquired a specific meaning in a given context. Thus, previous experience with the task to be learned and the information to be recalled appears to be as important a factor as the dose or the route of administration for testing the effect of VP. Concerning the latter, it seems that for the behaviorally active dose suited to the locus of treatment (peripheral or central), the effect of VP is the same, whatever the route of administration. Most studies on passive and active avoidance conditioning (Kovacs et al., 1986; De Wied et al., 1991) and social behavior (Le Moal et al., 1987; Dantzer et al., 1988) support this assumption. Concerning discriminative learning in the visual discrimination task, we found that subcutaneous, i.c.v., or intrahippocampal VP treatment alleviates the substantial forgetting shown after a

24-day time lapse. For this task, we also showed that the injection of the peptide before the first learning session deteriorates performance, regardless of whether the locus of the treatment is peripheral, i.c.v., or intrahippocampal. In contrast, we did not find the same result for the subcutaneous and intrahippocampal route of administration when testing the effect of the post-session injection of VP. When the peptide was injected after the first learning session, it markedly improved performance if the treatment was performed subcutaneously, whereas a weak deleterious effect was obtained if it was administered in the ventral hippocampus. The performance of the two groups at the time of treatment was similar, so this cannot explain the difference in the results. This discrepancy points out that in this particular case, the choice of a treatment route may be an important parameter. When the treatment is done via the general route, which involves broad diffusion of the peptide, the behavioral effect of VP is usually the same as for intra structure treatment. By contrast, when the treatment is performed in situ, which involves limited diffusion of the peptide within the injection site, the effect of VP is linked to the function of the structure. Indeed, studies on the central effects of VP have shown that the peptide can modulate learning and memory processes, depending on where in the brain it is applied (Kovacs et al., 1979; Veldhuis et al., 1987). Using a local application of vasopressin antiserum in several brain sites, Van Wimersma Greidanus and Boars (1993) showed that endogenous vasopressin in the habenular region plays a role in retrieval processes and not in storage processes for passive avoidance behavior. In contrast, they showed that vasopressin in the hippocampus (ventral and dorsal parts) is involved in retrieval as well as storage. For the hippocampus, we previously showed that the effect of VP was specific to each part of the structure. Indeed, VP in the ventral hippocampus was found to be involved in retrieval as well as relearning, while in the dorsal hippocampus, the peptide was only involved in retrieval processes. Concerning storage processes, Kovacs et al. (1986) and Veldhuis et al. (1987) showed that a post-training injection of VP enhances the retention of passive avoidance conditioning in rats. In our study, VP also improved information storage, but only when the information acquired was meaningful to the animal. This result is somewhat difficult to explain, especially if we consider recent studies on the activation of various brain structures during memory processes (Anonhin and Rose, 1991; Rose, 1991; Heurteaux et al., 1993). Of particular interest is the finding reported by Sif et al. (1991), according to whom both the dorsal and the ventral hippocampus display (^{14}C)-glucose labelling patterns 5 min after training in a spatial discrimination task. This labelling was found to be the same, no matter how well the behavioral task was learned. So, our results suggest that even if the hippocampus is mainly involved in memory consolidation, VP does not seem to participate in the modulation of the early stage of these processes, while being involved in the modulation of the later stages of consolidation. This assumption may also mean that a molecule which was endogenously present in a particular structure does not systematically participate in all of the steps of a given function.

Behavioral results obtained for a given brain area, especially the septal area and the hippocampal formation, in conjunction with what is already known about the various types of actions induced by the peptide, suggest that the differences observed between the behavioral effects of VP are due to the fact that the peptide's action depends on the functional involvement of the neuronal circuits upon which it acts. For the ventral hippocampus, we have shown that the facilitatory effects of VP on retrieval processes are dependent upon the functional state of the noradrenergic receptors. For the lateral septum, it has been shown that the involvement of vasopressin in social behaviors such as social recognition (Bluthé et al., 1990) depends upon the androgen level in the blood. The involvement in other social behaviors of testosterone-dependent vasopressinergic neurons in the medial amygdala and the lateral septum was also reported by Koolhaas et al. (1990, 1991). These data raise the question of whether there is a difference between the action of VP on social and non-social behavior. In the light of the fact that the vasopressinergic innervation of the ventral hippocampus, like the lateral septum, is dependent upon the blood androgen level (De Vries et al., 1985), it

would be worthwhile to determine whether such a dependency exists for the effects of VP on memory consolidation and retrieval of discriminative learning in this part of the hippocampus. Faiman et al. (1991) reported that the facilitating effect induced by vasopressin on the retention of an inhibitory avoidance response may be mediated by activation of the central nicotinic cholinergic mechanisms. Thus, it appears that VP may interact with various neurotransmitter systems. In order to better understand the links between the peptide and these neurotransmitter systems, their anatomical location and functional implications must be specified. This would help improve our understanding of the systems that interact during memory processes.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
Progress in Brain Research, Vol. 119

CHAPTER 5.9

Vasopressin metabolites: A link between vasopressin and memory?

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The effects of endogenous metabolites of the neuropeptide vasopressin (VP) in behavioural tests led to the hypothesis that VP metabolites have a more selective function than VP. In contrast to VP, no peripheral effects have been found thus far with VP metabolites and their function seems to be associated with memory-related behaviour. VP metabolites can improve both consolidation and retrieval of memory. Effects on autonomic and electrophysiological parameters and interactions with other neurotransmitter systems have provided some information about the processes that could underlie the effects of VP metabolites on memory-related behaviour. There is evidence that the effects of VP metabolites could be mediated by a VP metabolite receptor, which is different from the known VP receptors. The VP metabolite receptor could be a link between the neuropeptide VP and memory-related behaviour.

Introduction

The neuropeptide concept was formulated by De Wied at the end of the sixties (De Wied, 1969) and later reformulated (De Wied, 1987) as follows:

> Neuropeptides are endogenous substances present in nerve cells and involved in nervous system functions. They are formed following gene expression in the nerve cells. They are produced in large precursor molecules and several are formed from the same precursor molecule such as ACTH, α-MSH and β-endorphin from pro-opiomelanocortin. A cascade of processes evolves in peptidergic neurons to express the genetic information into biologically active neuropeptides. These processes determine the quantity of peptides synthesized as well as the nature of their biological activity through size, form and derivatization of the end product. In this way, sets of neuropeptides with different, opposite and more selective properties are formed from the same precursor.

Vasopressin (VP) is a peptide that fulfils the criteria for being a neuropeptide as formulated in the neuropeptide concept. VP has always been well known as a peripheral hormone. In the mid-sixties VP was, as one of the first peptides, found to have effects on central cognitive functioning (De Wied, 1965). With this finding the excitatory field of neuropeptide research was founded. The discovery in the beginning of the eighties that also the metabolites of VP have central functions added a new dimension to the field of VP research (Burbach et al., 1983). As discussed in this chapter, VP metabolites illustrate that not only presynaptic processing of neuropeptide precursors, but also subsequent extracellular processing of peptides within or outside the synapse can be crucial for enabling a neuropeptide to exert its physiological effect.

In this chapter original research articles, describing functional studies on VP metabolites (VP 4–9, [pGlu^4,Cyt^6]VP 4–9; VP 4–8, [pGlu^4,Cyt^6]VP 4–8; VP 5–9, [Cyt^6]VP 5–9); VP 5–8, [Cyt^6]VP 5–8), are reviewed in order to identify the function(s) of VP

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metabolites in the brain. The metabolism of VP, leading to formation of VP metabolites, is discussed in another chapter (Burbach, this volume). Studies on VP itself and its analogues (DG-VP, DD-VP) and fragments (C-terminal di- and tripeptides and the pressinamide ring) have already been reviewed (De Wied et al., 1993; see also this volume) and are not discussed in this chapter.

Vasopressin metabolites have effects on memory-related behaviour

Research on the functions of VP in the brain started with a publication, in which it was shown that the rapid extinction of active avoidance behaviour found in posterior lobectomized rats was counteracted by administration of a VP-containing extract of the pituitary (De Wied, 1965). After this finding many studies focused on the learning and memory enhancing effects of VP (for reviews see van Ree et al., 1978; van Wimersma Greidanus et al., 1986; De Wied et al., 1993). The passive avoidance test proved to be a sensitive bioassay for the central activities of VP and related peptides. Also the effects of VP metabolites on learning and memory processes have been studied mainly in this test (Burbach et al., 1983; De Wied et al., 1984a,b, 1987, 1991; Gaffori and De Wied, 1986; Kovacs et al., 1986, 1989; Lin et al., 1990; Hirate et al., 1997; Tanabe et al., 1997). Furthermore, effects of VP metabolites on memory-related behaviour have been investigated using maze tests, the social memory test and some other tests.

Conditioned avoidance tests

In one study VP metabolites decreased the extinction in a one-way active avoidance test (De Wied et al., 1987). All other conditioned avoidance studies used the passive avoidance test. The passive avoidance test consists of an acquisition trial, in which a rat receives a foot-shock after entering a box, and a test trial, in which the latency time to enter the box again is measured. The stronger the rat has associated the box with the foot-shock, the longer the latency time will be. Usually, the acquisition trial and test trial are separated by a 24 h interval. The passive avoidance test allows measurement of drug effects on consolidation (by administration before or just after the acquisition trial) and retrieval (by administration before the test trial).

After intracerebroventricular (i.c.v.) injection, VP metabolites facilitated both consolidation (Burbach et al., 1983) and retrieval (De Wied et al., 1987) of passive avoidance behaviour. VP metabolites were more potent than VP. For example, the minimum effective dose of VP 4–9 was as low as 0.03 pg, while 100 pg of VP was required to enhance consolidation (Burbach et al., 1983). Also after subcutaneous (s.c.) administration, VP metabolites were more potent than VP (De Wied et al., 1984a,b, 1987). Notably, VP 3–9 showed some activity but always with a lower potency than both VP and the shorter VP metabolites (De Wied et al., 1987).

There has been discussion whether the effects of VP and VP metabolites are mediated by structures in the brain or by peripheral organs. The central activity of VP metabolites was confirmed by the observation that about 1000 times more s.c. administered VP 4–8 was needed than with i.c.v. administered VP 4–8 (De Wied et al., 1984a). There are indications on which brain structures are involved. VP metabolites were effective when administered into the ventral or dorsal hippocampus or the dorsal raphe nucleus. The most pronounced effects were observed in the ventral hippocampus (Kovacs et al., 1986). In this brain region the effective dose of VP 5–8 was 100 times lower than with i.c.v. administration. This suggests that the ventral hippocampus may be an important target site for the effects of VP metabolites in avoidance tests.

Several analogues of VP metabolites have been shown to facilitate passive avoidance behaviour more potently than the original VP metabolite (Lin et al., 1990; Hirate et al., 1997; Tanabe et al., 1997). A cysteinyl-methyl-ester derivative of VP 4–8 was more potent than VP 4–8 (Lin et al., 1990). A VP 4–9 analogue with serine at the position of cysteine was more potent than VP 4–9, which correlated with a longer half-life of the VP 4–9 analogue in the blood (Hirate et al., 1997). A cationized VP 4–9 analogue was more potent than VP 4–9. This analogue had an arginyl-histidinyl-prolyl tripeptide attached to the cysteine, which

could have increased transport across the blood–brain-barrier. After reaching the extracellular space of the central nervous system (CNS) the VP 4–9 analogue was converted to VP 4–9 (Tanabe et al., 1997).

The behavioural effects of VP metabolites can be antagonized. Interestingly, the V_{1a} receptor antagonist $d(CH_2)_5Tyr(Me)VP$ inhibited the effects of VP 4–8 on passive avoidance in a competitive way, suggesting that the antagonist interferes with the VP 4–8 binding site involved in its effect (De Wied et al., 1984a,b). The effect of VP 4–8 on consolidation of passive avoidance behaviour was antagonized more potently by the oxytocin receptor antagonist $Des\text{-}Gly,(NH_2)^9\text{-}d(CH_2)_5[Tyr(Me)^2,Thr^4,Orn^8]$vasotocin than by the V_{1a} and V_2 receptor antagonists (De Wied et al., 1991). N^{α}-Acetyl-VP antagonized the effects of VP 5–9 on retrieval of passive avoidance behaviour, but showed little antagonistic activity for the effects of the whole nonapeptide VP on passive avoidance (Kovacs et al., 1989). Furthermore, a VP 4–8 analogue, with a 2-aspartyl substitute for the 2-asparaginyl residue in VP 4–8, could antagonize the effect of VP 4–8 on passive avoidance behaviour (Du, this volume). VP metabolites can reverse or prevent learning and memory deficits in the passive avoidance test. Pentylenetetrazol-induced retrograde amnesia was restored by VP and its metabolites, especially by VP 4–9 and VP 5–9 (De Wied et al., 1987). Cycloheximide-induced retrograde amnesia was prevented by VP 4–9 and VP 5–8 (Hirate et al., 1997).

Maze tests

Effects of VP metabolites were also found in maze tests. In the radial maze VP 4–9 improved consolidation in rats with a high error-baseline, but not in rats with a low error-baseline (Strupp, 1989). VP 4–9 restored scopolamine-induced disruption of radial maze learning after s.c., i.c.v. and local administration in the ventral, but not dorsal, hippocampus. Also VP 5–8 was effective, but not VP 6–8 and VP 5–7 (Fujiwara et al., 1997). VP 4–9 enhanced learning rate, final performance, working memory and reference memory in the radial maze when it was administered before each training-session on 6 consecutive days (Dietrich and Allen, 1997a). These enhancing effects were partly blocked by a lesion of the hippocampus (Dietrich and Allen, 1997b). The hole-board search task is comparable to the radial maze, except that holes in the floor of the apparatus instead of arms are baited. In this task, pretraining administration of VP 4–8 on 6 consecutive days enhanced both reference and working memory at a low, but not at a high dose (Vawter et al., 1997).

VP 4–9 s.c. administered before acquisition trials restored performance of rats impaired by prefrontal infra/prelimbic cortex lesions in the Morris maze (unpublished results). Chronic neonatal VP 4–8 facilitated acquisition and maintenance in a maze for brightness discrimination (Lin et al., 1990). Also a VP 5–8 analogue could improve performance in this test (Du, this volume). Chronic peripheral administration of VP 4–9 has been reported to increase the amount of errors in a spatial alternation task (McDaniel et al., 1989). Whether this is indicative of an impairing effect on memory processes is difficult to conclude, because it can also be argued that the original response in the VP 4–9-treated rats was better maintained. In conclusion, VP metabolites can improve performance in maze tests, especially in rats with a low baseline performance level.

Social memory test

VP metabolites affect the social memory of rats for conspecifics, as measured in the social memory test (Popik, this volume; Dantzer, this volume). Social memory was improved by s.c. administration of DG-VP, VP 4–8, VP 4–9, VP 5–9 or VP 5–8 (Popik et al., 1991; Sekiguchi et al., 1991). Also after local administration into the septum VP 4–8 and VP improved social memory (Popik et al., 1992). In contrast, administration of VP, VP 4–8 or VP 4–9 into the medial preoptic area had no effect (Popik and van Ree, 1991). VP fragments containing the covalent ring could extend social recognition over a period of 24 h using s.c. administration. This effect was not found with VP metabolites (Popik and van Ree, 1992).

Table 1
Time of administration of VP metabolites in tests for memory-related behaviour[a]

	Administration of VP metabolites				
	Before acquisition	After acquisition	Before test trial	Chronically	Combined with
Avoidance behaviour		+	+		
	+				CHX
			+		PTZ
Maze tests	+	+		+	
				+ (neonatal)	
			+		SCOP
				+	PFC or HIP lesion
Spatial alternation				−	
Social memory		+			
Conditioned freezing		0	+		
Self-administration				+/−	
Ethanol-tolerance				+	
Socially transmitted food preference			+/−		

[a] +, positive effect; − negative effect; 0, no effect. CHX, cycloheximide; PTZ, pentylenetetrazol; SCOP, scopolamine.

Other learning and memory tests

In a socially transmitted food-preference test an observer rat was exposed to a demonstrator rat, that had consumed flavoured food just before. After a retention interval the observer rat was s.c. injected with VP 4–9 and preference was measured for either the flavoured food, that also the demonstrator rat had consumed, or for food with a different flavour. VP 4–9 facilitated retrieval of socially transmitted food preference after a long retention interval, after which forgetting had occurred in the control group. In contrast, VP 4–9 impaired recall when administered after a short retention interval, after which forgetting had not yet occurred in the control group (Bunsey and Strupp, 1990; Strupp et al., 1990). In another test, VP 4–9 enhanced recall of conditioned freezing after s.c. administration prior to the test trial (Stoehr et al., 1992). VP metabolites also have effects on initiation of addiction, which can be seen as a form of memory. VP 4–8 decreased both heroin and cocaine intake during initiation of intravenous (i.v.) drug self-administration. However, VP 4–9 increased heroin intake (van Ree et al., 1988). VP 4–9 maintained functional ethanol tolerance in mice that were previously fed with an ethanol containing diet. This effect, which was blocked by a V_{1a} receptor antagonist, correlated with VP 4–9-induced c-fos expression in the septum (Szabo et al., 1991).

VP metabolites have effects on both consolidation and retrieval of memory

VP metabolites have been administered before and after the acquisition trial (in which something is learned), before the test trial (in which something has to be remembered), as well as chronically (Table 1). Effects of VP metabolites can be divided into effects on consolidation of memory and effects on retrieval of memory. Effects on consolidation take place during or shortly after the acquisition trial. Effects on retrieval take place during the test trial. The distinction between effects on consolidation and effects on retrieval can not be made with chronic administration. Only in the ethanol-toler-

ance study chronic treatment started after the tolerance was acquired, indicating an effect on retrieval. In all other studies chronic treatment started before the first acquisition trial, allowing effects on both consolidation and retrieval.

In three studies pre-acquisition administration was performed, but this was done s.c. within 60 min prior to the acquisition trial. Because the half-life of VP 4–9 in blood as measured in vitro is 40 min (Hirate et al., 1997), this allowed the VP metabolites to be effective not only during, but also directly after the acquisition trial. Effects of VP metabolites administered directly after the acquisition trial were found in studies using conditioned avoidance behaviour, maze tests and social memory. Especially the effects observed in the avoidance and maze tests point to a direct effect on consolidation, because the intervals between administration and test trial, 7.5 h in the maze test and up to 72 h in the avoidance test, were long enough for the VP metabolites to disappear before the test trial, hereby making direct effects on retrieval less likely. In the social recognition studies, effects of VP metabolites have been reported when the administration, directly after the acquisition trial, was 2 h before the test trial, but effects were also observed 4 h after administration (unpublished results).

Effects of VP metabolites on retrieval, by administration directly before the test trial, were observed in the passive avoidance test, conditioned freezing test and socially transmitted food preference test. Pre-test trial administration requires controls for non-specific effects during the test trial. In the passive-avoidance test it is important that there are no effects of the used doses on general activity. This has been shown for VP metabolites (Kovacs et al., 1989; Thurston et al., 1992). In the conditioned freezing test VP 4–9 was administered 1 h before test trial. The effective dose of VP 4–9 did not induce spontaneous freezing in naive rats, indicating that the effect on conditioned freezing was a specific effect on retrieval (Stoehr et al., 1992). In the socially transmitted food preference test VP 4–9 was administered 1 h before the test trial (Bunsey and Strupp, 1990; Strupp et al., 1990). The use of discrimination between flavoured diets during the test trial ensured that the VP metabolite effects on retrieval were not due to non-specific effects.

In conclusion, improving effects of VP metabolites have been found both on consolidation and retrieval of memory. Impairing effects of VP metabolites were probably on retrieval processes, because in these studies VP metabolites were administered just before the test trial (Bunsey and Strupp, 1990; Strupp et al., 1990) or chronically (McDaniel et al., 1989), which allows effects during the test trial.

Mechanisms underlying effects of VP metabolites on consolidation and retrieval

The mechanisms underlying the effects of VP metabolites on consolidation and retrieval of memory are still obscure. Some possible central mechanisms are offered by studies on autonomic effects, electrophysiological effects and effects of VP metabolites on other neurotransmitter systems. The results of these studies are summarized in Table 2, part A.

Are the effects of VP metabolites on consolidation and retrieval mediated by the same mechanisms or are different mechanisms involved? In one study (Tanabe et al., 1997), in which the same dose of an analogue of VP 4–9 was administered after the acquisition trial, before the test trial or at both time-points, the improving effects of all three treatments were the same. This was not due to a ceiling-effect, because improvement of performance was sub-maximal. That the effects of the double treatment did not sum up could point to similar mechanisms involved. An example of a process that could link the effects on both phases is sensitization of the endogenous VP system. Motor effects of VP were sensitized by an i.c.v. VP 4–9 injection 24 h earlier (Poulin et al., 1995). This effect was also found with some other VP analogues and VP itself. Sensitization could cause effects of post-acquisition trial administration actually taking place during the test trial, and thus on retrieval processes. An argument against this hypothesis is that the time-effect curves for improving passive avoidance performance and sensitization of the VP system are different. When VP metabolites were administered directly after the learning trial, which is 24 h before test trial, they

Table 2
2. Effects of VP metabolites compared with VP[a]

	VP metabolites	VP	References[b]
A. CENTRAL EFFECTS			
Autonomic system			
Blood-pressure and heart rate i.c.v.	0	+	1
Blood-pressure and heart rate NTS	−	−	2
Pineal gland NAT activity	+	+	3
Endotoxin hypothermia	0	−	4
Electrophysiology			
Lateral septum field potential	+	+	5
Ventral hippocampus neuron excitation	+	+	6
Glutamatergic transmission hippocampus	+	+	7
LTP in hippocampus	+	+	8
Ischemia damage in hippocampus	+	+	9
Firing rate suprachiasmatic neurons	0	+	10
Neurotransmitters			
ACh release	+	+	11
β-Endorphin in CSF	0	−	12
Norepinephrine-induced cAMP	0	+	13
VP motor effects sensitization	+	+	14
B. BEHAVIOURAL EFFECTS			
Memory-related			
Avoidance behaviour	+	+	15
Maze tests	+	+/−	16
Spatial alternation	−	ND	17
Social memory	+	+	18
Conditioned freezing	+	ND	19
Self-administration	+/−	−	20
Ethanol-tolerance	+	+	21
Socially transmitted food preference	+/−	ND	22
Not related to memory			
Attention	+	+	23
Locomotor activity	0	−	24
Scratching/grooming	0	+	25
Anti-nociception	0	+	26
Rearing	0	−	27
Defecation	0	−	28
Flank marking	0	+	29
Flank gland grooming	0	+	30
Tadpole locomotor activity	−	−	31
C. PERIPHERAL EFFECTS			
Blood pressure	0	+	32
Bradycardia	0	+	33

Table 2 (*continued*)

	VP metabolites	VP	References[b]
Antidiuresis	0	+	34
Uterus contraction	0	+	35
ACTH-release	0	+	36
CRH potentiation	0	+	37
α-MSH-release	0	0	38

[a] +, positive effect; −, negative effect; 0, no effect. ACh, acetylcholine; ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; CSF, cerebrospinal fluid; LTP, long-term potentiation; MSH, melanocyte-stimulating hormone; NAT, N-acetyltransferase; NTS, nucleus tractus solitarii; VP, vasopressin; ND, not done.

[b] References: [1] Noszczyk et al., 1993; [2] Brattstrom et al., 1989; [3] Stehle et al., 1991; [4] Burbach et al., 1983; [5] Urban and de Wied, 1986; [6] Urban and de Wied, 1986; [7] Rong et al., 1993; Chepkova et al., 1995; [8] Rong et al., 1993; [9] Tanaka et al., 1994; [10] Mihai et al., 1994; [11] Fujiwara et al., 1997; Maegawa et al., 1992; [12]Sweep et al., 1990; [13] Brinton and McEwen, 1989; [14] Poulin et al., 1995; [15] Burbach et al., 1983; de Wied et al., 1984a; de Wied et al., 1984b; Gaffori and de Wied, 1986; Kovacs et al., 1986; de Wied et al., 1987; Kovacs et al., 1989; Lin et al., 1990; de Wied et al., 1991; Hirate et al., 1997; Tanabe et al., 1997; [16] Strupp, 1989; Fujiwara et al., 1997; Dietrich and Allen, 1997; Vawter et al., 1997; Liu et al., 1990; [17] McDaniel et al., 1989; [18] Popik et al., 1991; Sekiguchi et al., 1991; Popik et al., 1992; Popik and van Ree, 1991; Popik and van Ree, 1992; [19] Stoehr et al., 1992; [20] van Ree et al., 1988; [21] Szabo et al., 1991; [22] Strupp et al., 1990; Bunsey and Strupp, 1990; [23] Bunsey et al., 1990; Meck, 1987; [24] Thurston et al., 1992; Kovacs et al., 1989; Dietrich and Allen, 1997; [25] Thurston et al., 1992; Kovacs et al., 1989; [26] Thurston et al., 1992; [27] Kovacs et al., 1989; [28] Kovacs et al., 1989; [29] Irvin et al., 1990; [30] Irvin et al., 1990; [31] Boyd, 1991; [32] Burbach et al., 1983; Lin et al., 1990; de Jong et al., 1985; [33] de Jong et al., 1985; [34] de Jong et al., 1985; Lin et al., 1990; [35] de Jong et al., 1985; [36] Burbach, 1987; [37] Burbach, 1987; [38]Burbach, 1987.

were more effective than when administered 6 h after the learning trial, which is 18 h before the test trial (Gaffori and De Wied, 1986). In contrast, sensitization of the VP system by VP is the same when VP is administered 24 and 18 h before testing (Poulin and Pittman, 1993). This suggests that VP metabolites had a direct effect on consolidation processes during a critical time period. However, it is still possible that the mechanism underlying the effect on consolidation bears similarity with the mechanism that underlies the effect on retrieval. Interestingly, administration of VP metabolites into the ventral hippocampus improved both consolidation and retrieval in the passive avoidance test (Kovacs et al., 1986). This indicates that both input and output stages of information processing in the hippocampus may be affected by VP metabolites.

A possible mechanism for direct effects on consolidation is suggested by the electrophysiological data, showing that glutamatergic neurotransmission in limbic areas is increased in the presence of VP metabolites. This increased neurotransmission could lead to improved encoding of information presented during the acquisition trial (Staubli et al., 1994), for example by improving long-term potentiation (Rong et al., 1993). It can be that there is an optimal level of neurotransmission increase, above which a ceiling effect diminishes subsequent encoding of a memory trace. This would cause an optimal dose-range for facilitating effects on consolidation by VP metabolites. Interestingly, VP 4–9 showed an inverted U-shaped dose–response curve for effects on consolidation of passive avoidance behaviour (Burbach et al., 1983; De Wied et al., 1987). Also prevention of cycloheximide-impaired passive avoidance behaviour by several VP metabolites, which could be an effect on consolidation, showed inverted U-shaped dose–response curves (Hirate et al., 1997). However, no impairing effects of VP metabolites on consolidation have been observed, which argues against a ceiling effect.

As discussed earlier, impairing effects on memory-related behaviour found with VP metabo-

lites were probably all on retrieval and not on consolidation processes. An explanation is provided by a possible acute effect of VP metabolites on arousal, explaining both positive and negative effects on retrieval by the inverted U-shaped relation between arousal, or amount of VP metabolite, and performance. This is illustrated by the effects of VP 4–9 on socially transmitted food preference. Inverted U-shaped dose-response curves were observed for VP 4–9 effects on retrieval, with low doses improving and high doses impairing performance (Strupp et al., 1990). Also in a passive avoidance study VP 4–9 and a VP 4–9 analogue both had an inverted U-shaped dose–response curve for their effects on retrieval (Tanabe et al., 1997). Effects on arousal could result in changes in attention during the test trial. In two studies effects of VP metabolites on attention have been observed (Meck, 1987; Bunsey et al., 1990). Possible effectors of the relationship between VP metabolites and arousal or attention have been discussed by Bunsey and co-workers (Bunsey and Strupp, 1990; Bunsey et al., 1990).

In conclusion, VP metabolites administered just after the acquisition trial could have long-term positive effects by improving consolidation processes, possibly by facilitation of long-term potentiation. When VP metabolites are administered just before the test trial, their acute effects on arousal and attention could act positively or negatively on the retrieval effect. The similarities between the mechanisms underlying the effects on consolidation and retrieval have still to be established.

VP metabolites have a function and a receptor different from VP

Effects of VP metabolites compared to VP

VP and VP metabolites seem to share their effects on memory. VP has, next to effects in memory tests, also effects in other behavioural tests. However, almost no effects of VP metabolites on non-memory-related behaviour have been reported. Effects that were found with VP, but not with VP 4–9, include motor suppression, scratching behaviour and antinociception after intrathecal administration (Thurston et al., 1992), and flank marking and flank gland grooming after microinjection in the septum or bed nucleus of the stria terminalis in the golden hamster (Irvin et al., 1990). Furthermore, VP 5–9 did not alter ambulation, rearing, grooming and defecation in an open field (Kovacs et al., 1989). Also VP 4–9 had no effect on ambulation in an open field, while the same dose enhanced both working and reference memory in the radial maze (Dietrich and Allen, 1997). In contrast, intraperitoneal administered VP 4–9 did mimic the effect of VP on locomotor activity in tadpoles (Boyd, 1991).

VP is most widely known for its peripheral effects. Therefore, VP metabolites have been studied for possible peripheral effects. Vasopressor activity has been investigated in the classical bioassay of Dekanski (Dekanski, 1952). Intravenous injection of 10 ng VP significantly increased blood pressure in this test. However, VP 4–8 in a dose range of 10–500 ng did not affect blood pressure. Pretreatment with this VP metabolite did not antagonize or potentiate the effect of VP in this test (Burbach et al., 1983). Even i.v. administration of more than 8000 ng VP 4–8 did not change blood pressure (Lin et al., 1990). The increase in blood pressure and bradycardia observed after s.c. administration of 0.3–3 mg VP was not observed with VP 4–8 in the same doses (de Jong et al., 1985). Antidiuretic activities were also absent with metabolites of VP (de Jong et al., 1985; Lin et al., 1990). The oxytocinergic activity of VP which is about 10% of that of oxytocin, as tested on uterus contraction in vitro, was absent with the tested VP metabolites (de Jong et al., 1985). In contrast to VP, VP metabolites did not affect the release of the adrenocorticotropic hormone and the melanocyte-stimulating hormone, nor did they potentiate the effect of the corticotropin-releasing hormone (Burbach, 1987). In conclusion, the experimental data on the peripheral effects of VP metabolites thus far indicate that these peptides lack the classical hormonal activities of VP and do not interfere with these activities of VP. It remains to be investigated whether VP metabolites occur in peripheral organs producing VP and/or have peripheral effects that are not exerted by VP.

Table 2 summarizes the effects of VP metabolites as described in the literature so far. To date no

peripheral activities of VP metabolites have been found. When the behavioural activities of VP metabolites are compared to those of VP, these seem to be restricted to memory-related behaviour. Whether the literature represents the actual profile of VP metabolite-effects is difficult to tell. It has to be mentioned that most publications focused on memory-related behaviour. Further research should investigate the selectivity of VP metabolites for memory-related behaviour.

The VP metabolite receptor(s)

VP metabolites do not seem to activate one of the classical VP receptors and there are indications that they have their own receptor, which is different from the known VP receptors (Burbach, this volume). Is there a possibility of multiple VP metabolite receptors? Although VP metabolites are presented in Table 2 as a homogeneous group, differences between the activities of different VP metabolites exist. Opposite effects on self-administration of heroin were observed for VP 4–8 and VP 4–9 and at the receptor-level VP 4–8 and VP 4–9 seem to differ in binding-sites and agonistic properties (Burbach, this volume). Furthermore, differences between VP metabolites were observed in the passive avoidance test, concerning their effectiveness on consolidation or retrieval. After s.c. administration VP 4–8 and VP 5–8 were more effective on consolidation than on retrieval. In contrast, VP 5–9 was more effective on retrieval than on consolidation (Gaffori and De Wied, 1986). When administered i.c.v., the relative potencies of metabolites to improve consolidation processes were VP 4–9 > VP 4–8 > VP 5–8 > VP 5–9. For retrieval processes the relative potencies were VP 5–9 > VP 4–9 > VP 4–8 > VP 5–8 (De Wied et al., 1987). The same results were obtained when VP metabolites were administered locally into the ventral hippocampus. In this study VP 5–9 was more effective on retrieval than on consolidation, while VP 5–8 was a bit more effective on consolidation than on retrieval (Kovacs et al., 1986). These findings suggest that the differential effects of VP metabolites on consolidation and retrieval depend on the presence of the N-terminal pGlu for potency on consolidation and the C-terminal $GlyNH_2$ for potency on retrieval. Whether these differences between VP metabolites are caused by multiple VP metabolite receptors has yet to be determined.

Does the VP metabolite receptor link VP and memory?

When the behavioural activities of VP metabolites are compared to those of VP, they seem to be restricted to memory-related behaviour. Furthermore, VP metabolites do not activate VP receptors, but may activate a VP metabolite receptor or perhaps multiple VP metabolite receptors. The VP metabolite receptor could be responsible for the different effects of VP metabolites and VP. It can be hypothesized that the activities shared by VP metabolites and VP are all mediated by VP metabolites. In that case VP should first be metabolized to act on the VP metabolite receptor, thereby affecting memory-related behaviour. However, there are observations that may argue against this. VP analogues with a protected N-terminus, which are resistant to conversion to C-terminal VP metabolites, were still able to improve memory functions (Walter et al., 1978), and amastatin, an aminopeptidase inhibitor, did not impair the memory-improving effect of VP in the passive avoidance (unpublished results). It should, however, be noted that amastatin does not completely block VP metabolism (Stark et al., 1989).

An alternative explanation for the overlap of VP and VP metabolite effects could be that VP acts directly on the VP metabolite receptor, without having to be metabolized first. It is not clear if VP can activate the VP metabolite receptor. In two studies VP did not compete for VP 4–8 (Du et al., 1994) and VP 4–9 (Jurzak et al., 1995) binding sites, while in one study inhibition of VP 4–9 binding by VP was observed (Brinton et al., 1986; Burbach, this volume).

Future research should tell whether VP has to activate directly or indirectly, after metabolism, the VP metabolite receptor in order to affect memory-related behaviour. Antagonists can only be used for this purpose when their selectivity is high enough. Effects of VP on passive avoidance behaviour are attenuated by antagonists for the V1,

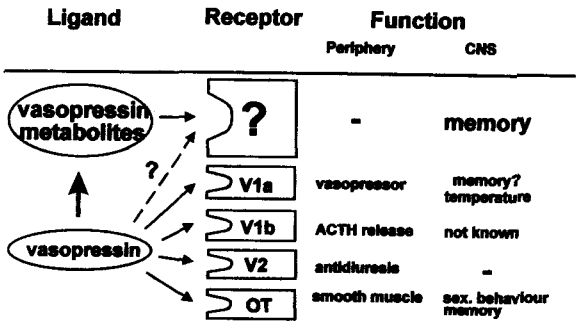

Fig. 1. How the VP metabolite receptor could link VP and memory.

V2 and oxytocin receptor (De Wied et al., 1984a,b, 1991; Kovacs et al., 1989). However, it could be that these antagonists also block the VP metabolite receptor. The antisense oligodeoxynucleotide method seems more specific. It has been shown that downregulation of the V_{1a} receptor by this method inhibits the positive effect of VP on social memory (Landgraf et al., 1995). This could mean that VP can affect memory-related behaviour independent from the VP metabolite receptor. At the end, cloning of the VP metabolite receptor and subsequently making a knockout of the VP metabolite receptor should tell whether the VP metabolite receptor is 'a' link or 'the' link between VP and memory.

A model of how the VP metabolite receptor could link VP and memory is presented in Fig. 1. An interesting example of the mechanism by which the VP metabolite receptor might link VP and memory is offered by another neuropeptide. Neuropeptide Y (NPY), but not NPY metabolites, acts at the postsynaptic receptor Y1, hereby acutely increasing food intake. In contrast, both NPY and NPY metabolites can activate the presynaptic receptor Y2, which results in enhanced consolidation in a T-maze (Flood and Morley, 1989).

Conclusions

VP metabolites are endogenous peptides with central actions, that are more selective than VP. Their effects seem to be associated with memory-related processes, especially consolidation and retrieval of memory. The effects of VP metabolites are probably mediated by their own receptor, which is different from the known VP receptors, and the existence of multiple VP metabolite receptors is possible. The VP metabolite receptor may link VP and memory, but it needs to be established whether the VP metabolite receptor is 'the' link, rather than 'a' link, between VP and memory.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 5.10

Vasopressin in the locus coeruleus and dorsal pontine tegmentum affects posture and vestibulospinal reflexes

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Vasopressin (VP) acts on both the locus coeruleus (LC) neurons and the neighbouring dorsal pontine reticular formation (PRF) neurons by exciting them. Experiments performed in precollicular decerebrate cats have shown that microinjection of 0.25×10^{-11} μg VP into the LC complex of one side increased the extensor rigidity of the ipsilateral limbs, while rigidity of the contralateral limbs remained unmodified or slightly decreased. The amplitude of modulation and thus the response gain of both the ipsilateral and the contralateral forelimb extensor triceps brachii to sinusoidal roll tilt of the animal (at 0.15 Hz, ±10°), leading to stimulation of labyrinth receptors, decreased significantly, while there was only a slight decrease in phase lead of the responses. These effects occurred 5–10 min after the injection, were fully developed within 30 min and disappeared in about 2 h. VP activation of presumed noradrenergic LC neurons had a facilitatory influence on ipsilateral limb extensor motoneurons, either directly through the coeruleospinal (CS) pathway, or indirectly by inhibiting the dorsal PRF and the related medullary inhibitory reticulospinal (RS) neurons. Moreover, because the facilitatory CS neurons fire out-of-phase with respect to the excitatory VS neurons, we postulated that the higher the firing rate of the CS neurons in the animal at rest, the greater the disfacilitation affecting the limb extensor motoneurons during side-down animal tilt. These motoneurons would then respond less efficiently to the excitatory VS volleys elicited for the same direction of animal orientation, leading to a reduced gain of the EMG responses of the forelimb extensors to labyrinth stimulation. In contrast to these findings, unilateral injections of the same dose of VP immediately ventral to the LC, i.e., in the peri-LCα and the surrounding dorsal PRF, where presumed cholinergic neurons are located, decreased extensor rigidity in the ipsilateral limbs while that of the contralateral limbs either decreased or increased. The same injection also produced either a moderate or a marked increase in gain of the multiunit EMG response of the ipsilateral triceps brachii to animal tilt. In the first instance the response gain of the contralateral triceps brachii to animal tilt increased slightly, while the corresponding response pattern remained unmodified, as shown for the ipsilateral responses (increased EMG activity during ipsilateral tilt and decreased activity during contralateral tilt). In the second instance, however, the response gain of the contralateral triceps brachii showed only slight changes, while the pattern of response was reversed. These effects occurred 5–20 min after the injection, developed fully within 20–60 min and disappeared in 2–3 h. We postulated that VP increased the discharge of the dorsal PRF neurons and the related medullary inhibitory RS neurons of the injected side, leading to reduced postural activity of the ipsilateral limbs. However, because these inhibitory RS neurons fire out-of-phase with respect to the excitatory VS neurons, it appeared that the higher the firing rate of the RS neurons in the animal at rest, the greater the disinhibition affecting the limb extensor motoneurons during ipsilateral tilt. These motoneurons would then respond more efficiently to the same excitatory VS volleys elicited by given parameters of stimulation, leading to an increased gain of the EMG responses. The contralateral effects could be attributed to crossed excitation by dorsal PRF neurons of one side, either of medullary inhibitory RS neurons or of excitatory CS neurons of the opposite side, respectively. We conclude that VP controls posture and gain of the VS reflex by acting on LC neurons as well as on dorsal PRF and the related medullary inhibitory RS neurons.

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Introduction

Immunohistochemical studies have shown that

[Arg]^8-vasopressin (VP), a nonapeptide which is present in the paraventricular and supraoptic hypothalamic nuclei (see Caffé et al., 1991), is transported by the hypothalamo-neurohypophyseal system and released into the blood as a hormone. In addition to this system, vasopressinergic fibres send extrahypothalamic projections to several brain structures (Swanson, 1977; Swanson and McKellar, 1979; Nilaver et al., 1980; Rosser et al., 1981; Sawchenko and Swanson, 1982; Buijs et al., 1983; Jenkins et al., 1984; Weindl and Sofroniew, 1985; Caffé et al., 1991), such as the locus coeruleus (LC) complex, the nucleus of the solitary tract, the dorsal nucleus of the vagus and the intermediolateral grey column of the spinal cord (Swanson, 1977; Nilaver et al., 1980; Sofroniew, 1980; Sawchenko and Swanson, 1982; De Vries and Buijs, 1983; De Vries et al., 1985). These findings, obtained with immunohistochemical techniques, correlated closely with the results of quantitative measurements, i.e., radioimmunoassays of VP and/or the related neurophysin in discrete regions of the rat and human brain, that showed high concentration of VP in the LC region (Hawtorn et al., 1984; Jenkins et al., 1984). The same region also contains VP receptors, as demonstrated by in vitro autoradiography (Phillips et al., 1988). Although it has been reported that the afferent input to the LC originates from the paraventricular nucleus of the hypothalamus (cf. Luiten et al., 1985), lesion experiments point to the bed nucleus of the stria terminalis as the main source of the vasopressinergic fibres in the rat dorsal pontine tegmentum (De Vries and Buijs, 1983; Sofroniew, 1985; Caverson et al., 1987; Caffè et al., 1991). Descending projections from the paraventricular nucleus and the bed nucleus of the stria terminalis, some of which terminate in the LC, were also studied by autoradiography in the cat (Holstege et al., 1985; Holstege, 1987).

The observation that descending vasopressinergic fibres project to the LC, where noradrenergic and noradrenaline (NA)-sensitive neurons are located (cf. Foote et al., 1983; Ennis and Aston-Jones, 1986), and possibly also to the neighbouring dorsal pontine reticular formations (PRF), where cholinergic (cf. Jones, 1991; Sakai, 1991) and cholino-sensitive (cf. Katayama et al., 1984; Barnes et al., 1987; D'Ascanio et al., 1988; Vanni-Mercier et al., 1989) neurons are located (cf. Pompeiano et al., 1987), is consistent with the fact that microiontophoretic application of VP exerts an excitatory influence on the corresponding neurons (Olpe and Baltzer, 1981).

One of the main roles attributed to the vasopressinergic system is the control of vegetative functions. For example, electrical stimulation of the supraoptic and paraventricular nuclei increased arterial blood pressure and heart rate in cats (Ciriello and Calaresu, 1980), and similar results were obtained after electrical stimulation of the LC (see Berecek et al. (1987) for references) or local injection of VP into this structure of conscious rats (Berecek et al., 1984). We have recently shown, however, that in addition to the regulation of autonomic functions, the LC complex also intervenes in the control of somatic functions (cf. Pompeiano, 1995). Stimulation (Fung et al., 1991) and lesion (D'Ascanio et al., 1985, 1989b) experiments have shown that this region, which includes the LC dorsalis (LCd), the LCα and the locus subcoeruleus (SC), exerts a facilitatory influence on limb extensor motoneurons. This effect is, at least partly mediated by the direct coeruleospinal (CS) projection which acts on limb extensor motoneurons either by exciting them (cf. Fung et al., 1991) or by suppressing the discharge of the inhibitory Renshaw cells linked with the corresponding motoneurons (Fung et al., 1987, 1988; cf. Fung et al., 1991). The noradrenergic LC complex neurons may also maintain postural activity by suppressing the discharge of dorsal pontine tegmental neurons (located in the peri-LCα and the dorsal PRF; cf. Pompeiano et al., 1991) which activate the medullary inhibitory reticulospinal (RS) neurons through a tegmento-reticular pathway (cf. Sakai et al., 1979; Jones and Yang, 1985).

The descending neural systems described above control not only posture, but also the gain of the vestibulospinal (VS) reflexes (cf. Pompeiano, 1995). Experiments performed in decerebrate cats have, in fact, shown that electrolytic lesion of the LC of one side (D'Ascanio et al., 1985), or functional inactivation of this structure elicited by local microinjection of the α_2-adrenergic agonist, clonidine, which leads to marked inhibition of the LC activity (Pompeiano et al., 1987), while it decreases

postural activity in the ipsilateral limb extensors, increases the gain of the multiunit electromyographic (EMG) responses of the triceps brachii to roll tilt of the animal leading to stimulation of labyrinth receptors. On the other hand, opposite results were obtained after local injection of the cholinergic agonist, carbachol, into the same structure, which activates the noradrenergic LC neurons (Stampacchia et al., 1987).

Since vasopressinergic afferents terminate not only on the LC complex neurons, but very likely also on the peri-LCα and the neighbouring dorsal PRF, and since the noradrenergic LC neurons suppress the discharge of these latter structures, experiments were performed to investigate: (1) whether activation of the LC and SC neurons, elicited by local microinjection of minute doses of VP into the LC complex, could modify posture as well as the gain of the VS reflexes elicited by sinusoidal stimulation of labyrinth receptors, and if so, (2) whether increased discharge of neurons located in the peri-LCα and the neighbouring dorsal pRF following local application of VP would produce postural and reflex changes opposite to those elicited by local application of VP in the LC.

The results of the experiments reviewed in this chapter have been reported in detail previously (Andre et al., 1992a,b).

Experimental procedures

The experiments were performed in precollicular decerebrate cats, which showed a symmetric rigidity of the forelimbs. Postural tonus was examined by evaluating the resistance which occurred during the stretch reflex produced by slow passive flexion of the forelimbs. The VS reflexes were tested by recording the multiunit EMG responses of the medial head of the triceps brachii to sinusoidal roll tilt of the animal at 0.15 Hz, $\pm$ 10°, as described previously (Manzoni et al., 1983a). In particular, sequential pulse density histograms (SPDHs) were obtained by averaging data from six sweeps, each containing the responses of two successive cycles (128 bins, with a bin width of 0.1 s). These stimulation sequences were repeated at regular intervals of 4–8 min for 1–3 h, before and after microinjection of minute doses of VP into the dorsal pontine tegmentum. The digital data related to the averaged responses (AR) were processed on-line with a computer system which performed a fast Fourier transform, and the results were used for statistical evaluations. In particular, the gain of the first harmonic component of the EMG responses, expressed in impulses/s per deg, and the phase angle of the responses expressed in degrees relative to the peak of the side-down displacement of the whole animal (downward rotation of the table towards the side of recording), were evaluated. The base frequency (BF, in impulses/s) was also evaluated and corresponded to the DC value obtained from the harmonic analysis of the responses, being comparable to the mean frequency of the multiunit discharge recorded at rest. VP (Sigma, St. Louis, USA) was injected stereotaxically through a stainless steel cannula, with a tip diameter of 300 μm, either into the LC complex of one side (at P2.5, L2.5 or 2.8, H-2.0 or -2.5) or into the dorsal PRF (at P2.5, L2.5 or 2.8, H-2.5 or -3.5). Usually, 0.25 μl VP solution (10^{-11} μg/μl saline, 0.9% NaCl) containing 5% Pontamine Sky Blue (Gurr, Poole, UK) as a marker was administered. The injected dose (0.25×10^{-11} μg VP), which turned out to be active to produce changes in posture as well as in gain of the VS reflexes, was close to the minimal dose used by previous investigators, who produced postural asymmetries in rats following intracisternal injection of VP (Vartanyan et al., 1989). The pH of the solution was adjusted with NaOH (0.5 N) or HCl (0.5 N) to reach the value of 7.4 $\pm$ 0.2. Each injection usually lasted 90 s. The EMG activity of the triceps brachii of both sides as well as the arterial blood pressure were recorded polygraphically. The injected spot was identified at the end of each experiment on serial frozen sections of the brainstem counterstained with Neutral Red.

Results

Postural changes after unilateral microinjection of VP either in the LC complex or in dorsal pontine reticular structures

Microinjection of 0.25×10^{-11} μg VP into the LC complex (i.e., LC and SC) of one side produced a postural asymmetry characterized by an increase

in extensor tonus in the ipsilateral limbs, while the decerebrate rigidity either remained unmodified or decreased slightly in the contralateral limbs. Similarly, spontaneous EMG activity as well as the myotatic stretch reflex increased in the medial head of the triceps brachii ipsilateral to the side of the injection. The same injection also produced a transient and slight increase in arterial blood pressure and heart rate, followed by a depressor response characterized by a decrease in blood pressure and heart rate.

Other experiments, however, have shown that microinjection of the same dose of VP in the dorsal PRF decreased the extensor tonus in the ipsilateral limbs, while tonus in the contralateral limbs either decreased slightly in a first group of experiments, or increased in a second group. These postural changes were also associated with parallel changes in spontaneous EMG activity as well as in the myotatic stretch reflex of the triceps brachii recorded both ipsilaterally and contralaterally to the side of the injection. No clear changes in blood pressure and heart rate were observed after local administration of the neuropeptide in the dorsal PRF. The VP-induced changes in posture and stretch reflex described above usually occurred within 5–10 min, reached a peak 30–40 min after the injection, and persisted for about 2 h, before disappearing gradually in 2–3 h.

Decrease in the response gain of a forelimb extensor of both sides to sinusoidal stimulation of labyrinth receptors after unilateral injection of VP in the LC complex

Sinusoidal modulation of the multiunit EMG activity of the medial head of the triceps brachii occurred during sinusoidal roll tilt of the animal at 0.15 Hz, ±10°, leading to stimulation of labyrinth receptors (Fig. 1A). Activity increased particularly during side-down, and decreased during side-up tilt (α-responses). The peak of the first harmonic responses was related to the extreme animal displacement, thus being attributable to stimulation of position-sensitive macular (utricular) receptors (see Manzoni et al., 1983a). Moreover, the gain of these responses elicited with the stimulation parameters detailed above was usually small in the decerebrate preparation (see Manzoni et al., 1983a; cf. Pompeiano et al., 1991). In order to compensate for the slight postural asymmetry which occurred after injection of VP into the LC of one side, the limb position was adjusted by decreasing or increasing the static stretch of the muscle, so that the spontaneous EMG activity remained constant throughout the experiment. The main result of these experiments was that the amplitude of modulation, and thus the mean response gain of the ipsilateral as well as of the contralateral triceps brachii to labyrinth stimulation, decreased significantly (t-test, difference between the means, $P < 0.001$) after microinjection of 0.25×10^{-11} µg VP into the LC complex of one side (Fig. 1A,B). This effect, which was found in 4 experiments, was due to both decreased discharge of the active motor units and reduced recruitment of motor units. It occurred even in the absence of any significant change in BF, thus leading to a decrease in response sensitivity (percentage change of the mean discharge rate per degree of displacement). The decrease in the response gain of the ipsilateral as well as of the contralateral triceps brachii to animal tilt was, in some instances, so prominent that no modulation of the multiunit EMG activity of the triceps brachii was observed after injection of VP into the LC of one side (Fig. 2A,B). In contrast to these clear-cut changes in gain, there was only a slight decrease in phase lead of the responses relative to animal position.

The effects described above began 5–10 min after the injection of VP and became most prominent within the first 30 min post-injection; the effects were followed for about 2 h before their disappearance. Fig. 2A shows that, in one experiment, the decrease in the response gain of the right triceps to animal tilt following two successive injections of VP in the ipsilateral LC complex recovered almost completely within about 2 h after the injection. The time course of the reduced gain of the responses of the contralateral limb extensor to animal tilt (Fig. 2B) was comparable to that obtained from the ipsilateral limb extensor (Fig. 2A).

The effects described above were not due to irritation phenomena after injection of the fluid, since there were changes neither in posture nor response

gain of the triceps brachii of both sides to labyrinth stimulation after a previous injection of an equal volume of saline stained with 5% Pontamine Sky Blue into the LC complex of that side. The results

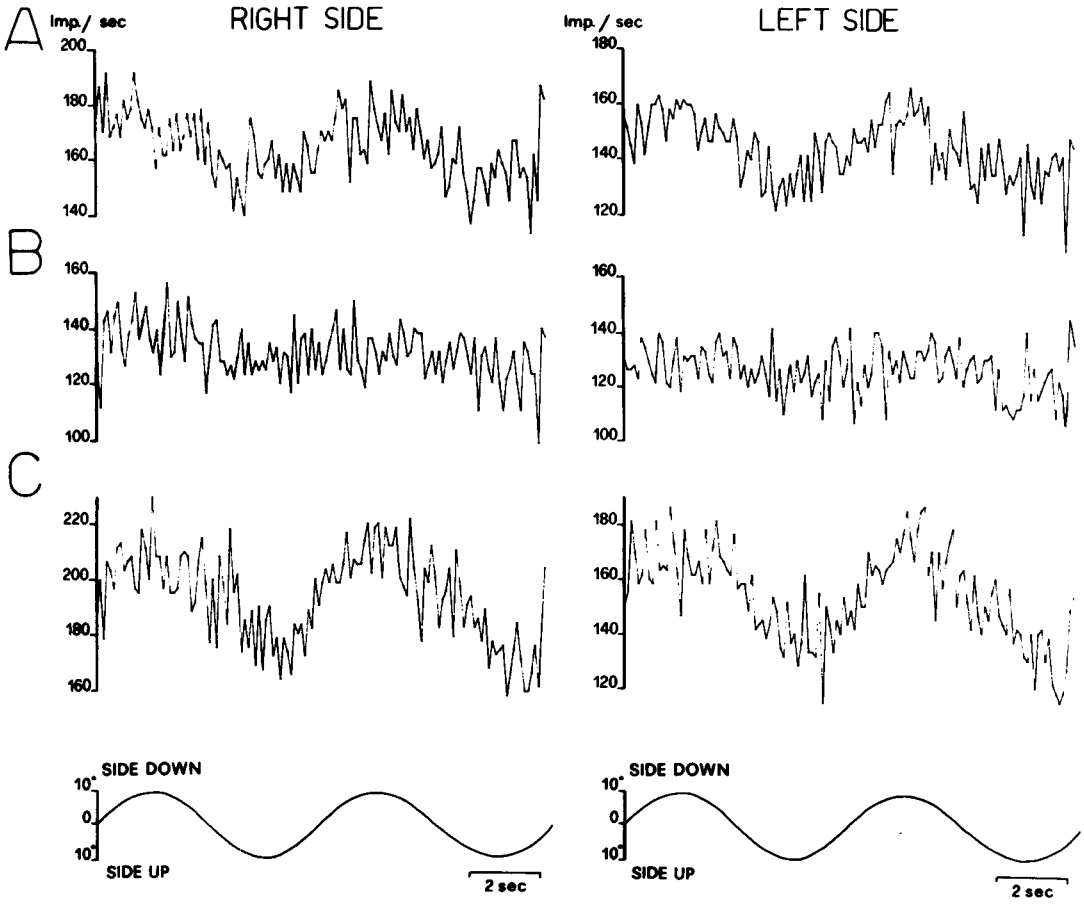


Fig. 1. Effects of local injections of VP in the LC complex of one side followed by injection of the same agent in the dorsal PRF of the opposite side. Precollicular decerebrate cat (Exp. 27). Sequential pulse density histograms (SPDHs) showing the multiunit averaged responses (AR) of the triceps brachii of both sides to sinusoidal roll tilt of the animal at 0.15.Hz, ±10° (average of 6 sweeps, using 128 bins with 0.1 s bin width). The lower trace in each column indicates animal displacement. Side-down means displacement of the animal towards the side of recording. (A) SPDHs recorded 23 and 30 min, respectively, before VP injections into the LC complex. The mean BF for the AR of the right muscle was 164.4 impulses/s, the gain was 1.09 impulses/s per deg, while the phase angle corresponded to a lead of +20.6° with respect to the peak of the ipsilateral side-down tilt. The values for the AR of the left muscle were: BF 144.1 impulses/s, gain 1.16 impulses/s/deg, phase lead +12.4°. (B) SPDHs recorded 61 and 89 min, respectively, after a first local injection of 0.25 μl VP solution (10^{-11} μg/μl saline) in the LC-SC area of the right side (I), followed by a second injection into the same area (II) at the time interval indicated in Fig. 2. On this side, the mean BF was 131.5 impulses/s, the gain was 0.53 impulses/s per deg and the phase lead +0.9°. On the left side the values were: BF 126.1 impulses/s, gain 0.50 impulses/s per deg, phase lag -9.0°. (C) SPDHs recorded 135 and 142 min, respectively, after the first injection (I) and 15 and 22 min after injection of 0.25 μl VP solution (10^{-11} μg/μl saline) in the dorsolateral part of the PRF of the left side (III), as indicated in Fig. 2. On the right side the mean BF was 193.1 impulses/s, the gain was 1.73 impulses/s per deg and the phase lag −7.4°. On the left side the values were: 154.1 impulses/s, gain 1.85 impulses/s/deg, phase lag −11.0°. From Andre et al. (1992a).

obtained, therefore, depended on injection of the neuropeptide.

Increase in the response gain of a forelimb extensor of both sides to sinusoidal stimulation of labyrinth receptors after unilateral injection of VP in dorsal pontine reticular structures

In order to compensate for the decrease in spontaneous EMG activity of the ipsilateral forelimb extensor following injection of VP into the dorsal PRF of one side, the corresponding limb was passively flexed to increase the static stretch of the muscle, thus leading to muscular activity comparable on the average to that obtained prior to the injection. An increase or a decrease of the static stretch of the muscle contralateral to the injected side was applied in order to maintain the spontaneous EMG activity constant throughout the experiment.

In a first group of experiments ($n = 3$), microinjection of 0.25×10^{-11} μg VP in the dorsal PRF of one side significantly increased the amplitude of the modulation and thus the response gain of the ipsilateral and to a lesser extent of the contralateral triceps brachii to given parameters of animal tilt (0.15 Hz, ±10°). This effect, which was due to both an increased discharge of the active motor units and increased recruitment of motor units, occurred even in the absence of any change in BF, thus leading to an increase in response sensitivity.

Although the BF was not greatly modified with respect to the value obtained prior to the injection, the gain of the responses after VP injection in the experiments reported above increased on the average to 190.3% of the control values in the ipsilateral muscle (t-test between means, $P < 0.001$) and to 150.9% of the control values in the contralateral muscle (t-test between means, $P < 0.01$). As to the phase angle of the responses, which was closely related to the peak of the side-down animal position (α-response), there was only a slight decrease in phase lead of the ipsilateral responses (corresponding on the average to −7.7°), which was associated with a slight increase in phase lead of the contralateral responses (corresponding on the average to +14.2°). The effects described above appeared 5–20 min after injection of VP in the dorsal PRF and reached their highest values in 30–60 min. The control values were returned within 2–3 h after the injection. An example of these multiunit EMG responses is shown in Fig. 3A,B (injection I).

In addition to these experiments, in which unilateral administration of VP into the dorsal PRF produced only a moderate and bilateral increase in gain not associated with significant changes in the phase angle of the EMG responses of the triceps brachii to animal tilt, there was a second group of experiments ($n = 5$) in which microinjection of the same dose of VP in the PRF of one side greatly increased the response gain of the ipsilateral muscle to animal tilt. Although, in these instances, the BF showed only slight changes with respect to the values obtained prior to the injection, the gain of the responses recorded after VP injection increased on an average to 296.3% of the control value in the ipsilateral muscle (t-test between the means, $P < 0.001$). These changes were associated only with a slight but non-significant decrease in phase lead of the ipsilateral responses, which were always related to the ipsilateral side-down tilt (α-responses). However, the contralateral triceps brachii showed a slight but insignificant decrease of the response gain which was often accompanied by a reversal of the response pattern, characterized now by an increased activity during contralateral tilt (β-responses). In these instances, the peak of the response showed an average phase lag of 172.6 ± 31.7° (SD) with respect to the ipsilateral side-down tilt. An example of these multiunit responses is given in Fig. 4A,B.

The increased gain of the EMG responses of the ipsilateral triceps brachii to animal tilt associated with reversal of the response pattern of the contralateral limb appeared 5–10 min after the injection, i.e., a shorter period than that in the first group of experiments reported on in this section. The highest values were reached in 20–30 min, and slowly returned to the control levels 2–3 h after the injection.

The effects induced by local injection of VP into the pRF were not due to irritation following injection of the fluid, since there were changes neither in posture nor response gain of the triceps brachii of both sides to labyrinth stimulation after a previous

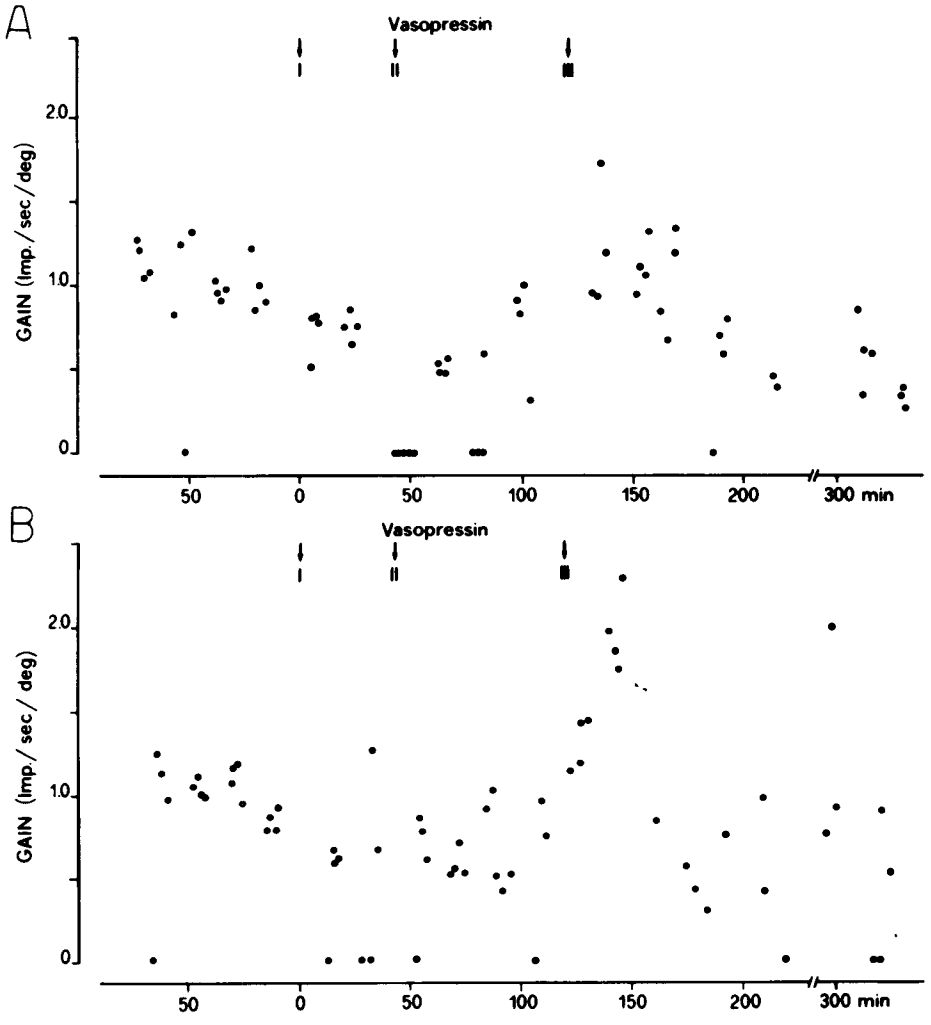


Fig. 2. Bilateral decrease in response gain of the triceps brachii to animal tilt after local injections of VP in the LC complex of one side, followed by a sudden and bilateral increase in gain after a local injection of the same agent in the PRF of the opposite side. Same experiment as in Fig. 1. The gain of the AR of the right (A) and left (B) triceps brachili to animal tilt at 0.15 Hz, $\pm 10°$ was evaluated and plotted at different time intervals before and after two injections in the LC complex of the right side of 0.25 μl VP solution (10^{-11} μg/μl saline), as indicated by the arrows (I and II). The areas which, upon injections, decreased the response gain of the triceps brachii of both sides corresponded to the ventral part of the LC and the SC area of the right side (see Fig. 5A). Injection of the same dose of VP in the dorsolateral part of the PRF of the left side (III) enhanced the response gain of the same muscle of both sides to animal tilt. These effects were not associated with significant changes in the phase angle of the responses (not shown). From Andre et al. (1992a).

injection of an equal volume of saline with 5% Pontamine Sky Blue into the PRF of that side (cf. also Barnes et al., 1987; D'Ascanio et al., 1988).

Localization of the injection sites

The neurons whose selective activation follow-

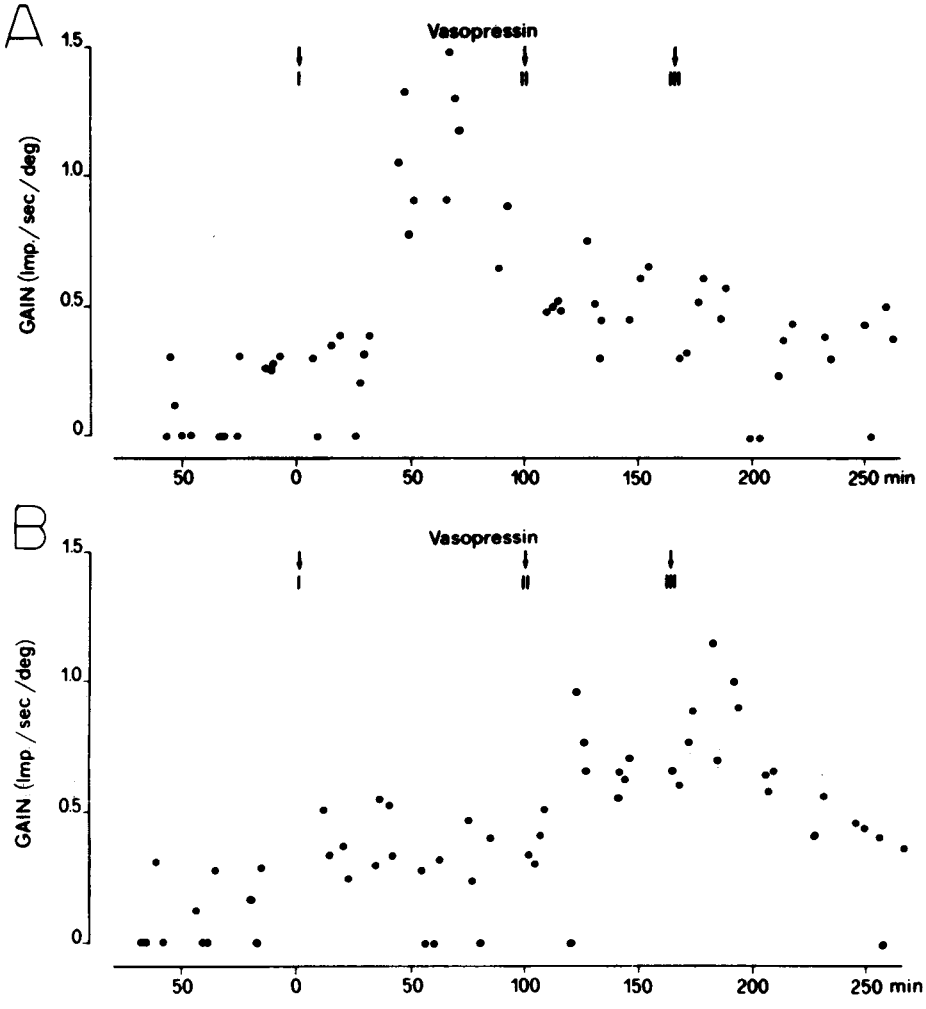


Fig. 3. Changes in response gain of the triceps brachii of both sides to animal tilt after local injection of VP in the dorsal PRF of one side, followed by injections of the same agent in the dorsal PRF of the opposite side. Precollicular decerebrate cat (Exp. 24). The gain of the AR of the right (A) and left (B) triceps brachii to animal tilt at 0.15 Hz, $\pm 10°$ were evaluated and plotted at different time intervals before and after injections in the dorsal pontine tegmentum on the right (I) and left (II and III) side of 0.25 µl VP solution (10^{-11} µg/µl saline), as indicated by the arrows. The first injection of VP (see Fig. 5B), particularly increased the response gain of the ipsilateral right triceps brachii to animal tilt, an effect which was followed by slow decay. Similarly, injections of the same dose of VP in the dorsolateral part of the pontine tegmentum of the left side (II and III) (see Fig. 5B) produced prominent and sudden increases in the response gain of the corresponding left muscle to animal tilt, followed by slow decay. In all instances the response gain of the triceps brachii contralateral to the side of the injection increased only to a smaller extent. The changes in the response gain described above were not associated with changes in the phase angle of the responses. From Andre et al. (1992b).

ing injection of VP increased postural activity in the ipsilateral limbs, while decreasing the gain of the EMG responses of the triceps brachii of both sides to sinusoidal stimulation of labyrinth receptors, were located in the LC complex which included both the LCd and the LCα (Exp. 15,II in Fig.

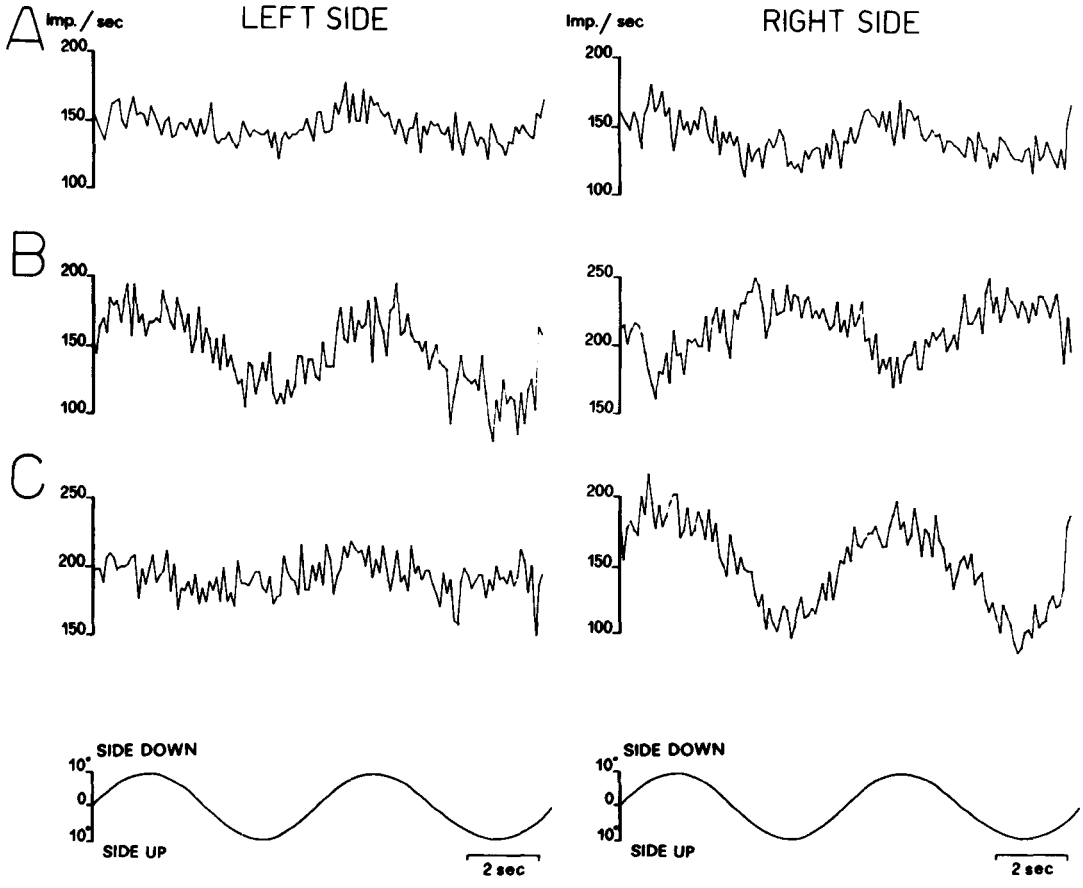
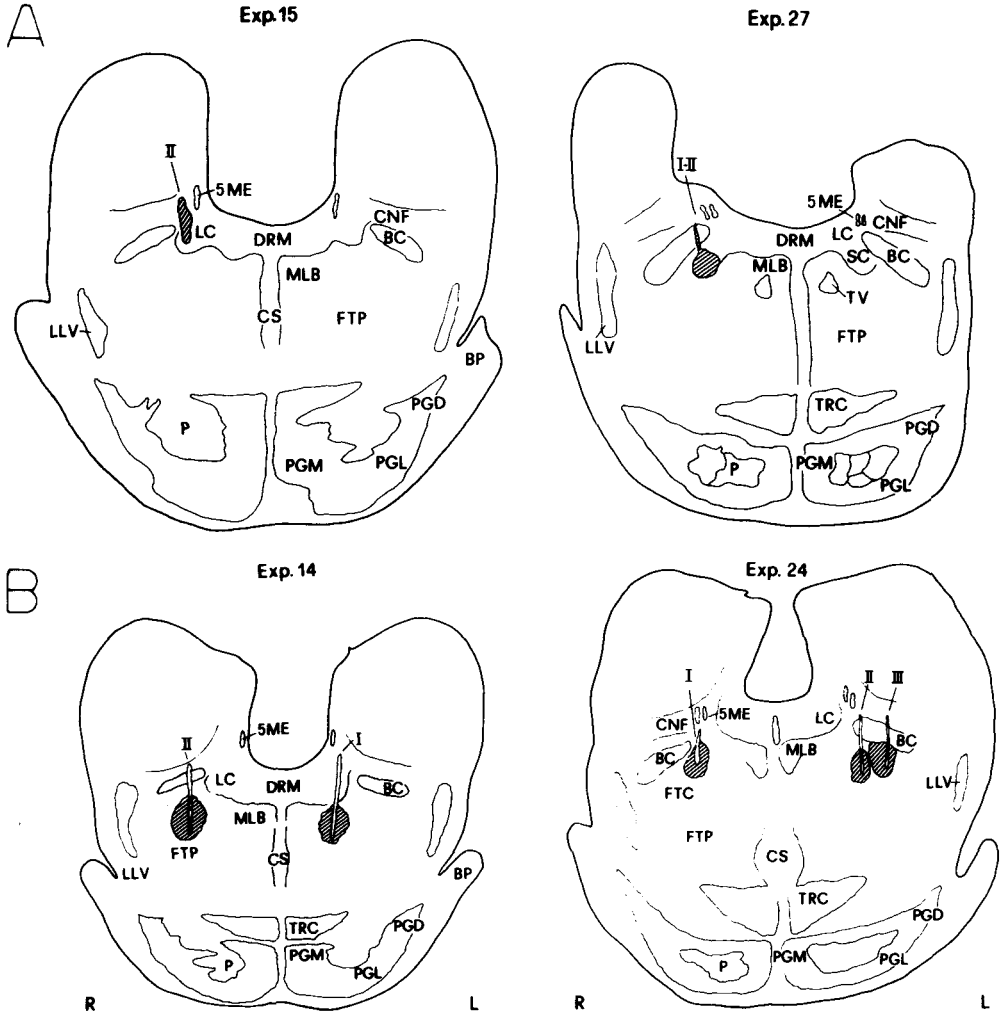


Fig. 4. Changes in the response pattern of the triceps brachii of one side to animal tilt after successive injections of VP in the dorsal pontine tegmentum of both sides. Precollicular decerebrate cat (Exp. 14). SPDHs showing the AR of the triceps brachii of both sides to animal tilt at 0.15 Hz, $\pm 10°$ (average of 6 sweeps, using 128 bins with 0.1 s bin width). (A) SPDHs recorded 159 and 18 min, respectively, before VP injection. The mean BF evaluated for the AR of the left muscle was 146.1 impulses/s, the gain was 0.99 impulses/s per deg, while the phase angle corresponded to a lead of $+30.2°$ with respect to the peak of the ipsilateral side-down tilt. The values for the AR of the right muscle were: BF 140.1 impulses/s, gain 1.45 impulses/s per deg, phase lead of $+19.9°$. (B) SPDHs recorded 32 and 55 min, respectively, after a first local injection of 0.25 μl VP solution (10^{-11} μg/μl saline) in the left pRF (I) (see Fig. 5B). On this side the mean BF was 144.7 impulses/s, the gain was 2.99 impulses/s per deg and the phase lag $-0.1°$ (α-response). On the right side the values were: BF 214.4 impulses/s, gain 2.15 impulses/s per deg, while the response pattern reversed (β-response) and showed a phase lag of $-164.0°$. (C) SPDHs recorded 69 and 65 min, respectively, after a second injection of 0.25 μl VP solution (10^{-11} μg/μl saline) in the right pRF (II) (see Fig. 5B). On the left side the mean BF was 191.2 impulses/s, the gain was 0.90 impulses/s per deg and the phase lead $+45.0°$. On the right side the values were: BF 149.8 impulses/s, gain 3.93 impulses/s per deg, while the response pattern reversed with respect to the previous record in (B) from a β- to an α-pattern and showed a phase lead of $+11.0°$. From Andre et al. (1992b).

5A). It is to be noted that only a small portion of the LC, mainly at the caudal level, was injected, as documented by the Pontamine Sky Blue dye staining of histological serial sections of the dorsal pontine tegmentum. In some instances the injection also affected the SC (Exp. 27,I–II in Fig. 5A). On the other hand, the structures whose selective activation by local injection of VP decreased postural

activity in the ipsilateral limbs, while it increased the gain of the EMG responses of the ipsilateral triceps brachii to labyrinth stimulation, were located in the dorsal pontine tegmentum and included the peri-LCα and PRF immediately ventral to it (Exp. 24,I–III in Fig. 5B). For contralateral responses, VP injection reversed the response pattern on the contralateral side in some experiments (Exp. 14,I in Fig. 5B) but not in others (Exp. 24,I in Fig. 5B). In both instances the injections were located in the dorsal part of the pontine tegmentum, but not at the same lateral level. While in the former experiments (see Exp. 14,I) the injected area was located ventromedially to the LC at the level of the dorsomedial part of the paralemniscal tegmental field (FTP), in the latter experiment the injected area involved the locus subcoeruleus as well as the dorsolateral part of the central tegmental field (FTC) close to the brachium conjunctivum (see Exp. 24,I). A comparison of all the histological findings indicates clearly that the different types of VP-induced effects described above were related to slight displacements of the injection sites, which involved either the dorsomedial or the dorsolateral part of the PRF.

It is of interest that microinjections of the same



dose of VP into several structures like the cuneiform nucleus, the marginal nucleus of the brachium conjunctivum, the medial longitudinal bundle and the contiguous medial area of the PRF were ineffective.

Effects of VP injection in dorsal pontine reticular structures of one side after administration of the same agent either in the LC or in dorsal pontine reticular structures of the opposite side

Since injection of VP into the dorsal PRF of one side increased the response gain of the triceps brachii to labyrinth stimulation, attempts were made to determine whether a PRF injection could antagonize the opposite effect induced by previous administration of the same agent into the LC complex of the opposite side. In the experiment illustrated in Fig. 1, the gain of the EMG responses of the triceps brachii of both sides to animal tilt, which corresponded on the average to 1.12 impulses/s per deg in the control situation (A), decreased bilaterally to 0.51 impulses/s per deg after local injection into the right LC complex of 0.25×10^{-11} μg VP (B). The amplitude of modulation recovered, and actually increased to values higher than those of the controls (i.e., 1.79 impulses/s per deg) following subsequent injection of an equal dose of VP into the left dorsal PRF (C). Fig. 2 illustrates, from the same experiment, the time course of the overwhelming facilitatory influences of VP injection into the PRF of one side (III) with respect to the opposite effects previously obtained by injecting the neuropeptide into the LC of the opposite side (I and II).

Another example of the site specificity of the effects is shown in Exp. 14, in which a first injection of VP into the dorsomedial part of the left PRF increased the response gain of the corresponding triceps brachii to animal tilt on an average from 0.99 to 2.90 impulses/s/deg, while the pattern of response to labyrinth stimulation remained of the α-type. On the contralateral (right) side, however, the response gain remained almost unmodified, since it changed on an average only from 1.30 to 1.29 impulses/s/deg, but the response pattern reversed from the α- to the β-type. A second injection of the same dose of VP into the dorsolateral part of the right PRF led to the reappearance of α-responses in the corresponding triceps brachii, whose gain increased on the average to 2.92 impulses/s per deg; on the other hand, the response gain of the left triceps brachii decreased on an average to 1.70 impulses/s per deg, while the response pattern remained of the α-type. Representative responses of the triceps brachii of both sides to animal tilt, recorded in this experiment, are shown in Fig 4A–C.

Fig. 5. Schemes showing the localization of VP injections in either the LC complex (A) or the dorsal pontine tegmentum (B). Precollicular decerebrate cats. Shaded area indicate the spread of the injected solution stained with 5% Pontamine Sky Blue. L, left side; R, right side of the animal. (A) Exp. 15: injection within the LC of the right side of 0.25 μl VP solution (10^{-11} μg/μl saline) (II) decreased the response gain of both the ipsilateral and the contralateral triceps brachii to animal tilt at 0.15 Hz, ±10°. Exp. 27: in this experiment two injections within the ventral part of the LC and the SC area of the right side of a VP solution at the same concentration indicated above (I–II) decreased the response gain of the triceps brachii of both sides to animal tilt (see Figs. 1 and 2). (B) Exp. 14: injection within the dorsomedial part of the left pRF of 0.25 μl VP solution (10^{-11} μg/μl saline) (I) enhanced the response gain of the left triceps brachii and reversed from the α- to the β-type the response pattern of the right triceps brachii to animal tilt at 0.15 Hz, ±10° (see Fig. 4A,B). Injection within the dorsolateral part of the right PRF of the same dose of VP (II), while decreasing the response gain of the left triceps brachii, increased the gain and abolished the reversal of the response pattern of the right triceps brachii to animal tilt at the same parameters indicated above (see Fig. 4C). Exp. 24: injection into the dorsolateral part of the right PRF of a VP solution at the same concentration as indicated above (I) enhanced the response gain of the ipsilateral and to a lesser extent also of the contralateral triceps brachii to animal tilt (see Fig. 3A,B, respectively). Similarly, two successive injections into the dorsolateral part of the left pRF of the same dose of VP (II and III) enhanced the response gain of the ipsilateral, and to a lesser extent, also of the contralateral triceps brachii to animal tilt (see Fig. 3B,A, respectively). In contrast to the previous experiment, the phase angle of these responses remained almost unmodified. Main abbreviations: BC, brachium conjunctivum; CNF, cuneiform nucleus; FTC, central tegmental field; FTP, paralemnical tegmental field; LC, locus coeruleus; MLB, medial longitudinal bundle; SC, locus subcoeruleus; 5ME, mesencephalic trigeminal nucleus. From Andre et al. (1992a,b).

Discussion

As reported in the Introduction, VP-containing fibres originating from hypothalamic nuclei project to the LC region (cf. Buijs et al., 1983; Weindl and Sofroniew, 1985; Caffé et al., 1991). In particular, these VP descending fibres terminate in the dendritic field of the LC which surrounds the cytoarchitectonically defined nucleus containing its perikarya (Caffé et al., 1991). This finding explains why it could be shown, using in vitro autoradiography, that the LC was apparently free of VP binding sites, which were found, however, in the SC region (Phillips et al., 1988). VP descending fibres also terminate in the periventricular tegmentum (see also De Vries and Buijs, 1983). Since, in cats, noradrenergic and cholinergic neurons are intermixed in overlapping regions between the ventral part of the LC and the dorsal part of the PRF (cf. Jones, 1991; Sakai, 1991) and since both populations of neurons contribute to the dendritic field of this tegmental region, we postulated that the VP-containing fibres terminate, not only on noradrenergic and NA-sensitive (cf. Foote et al., 1983) LC neurons particularly located in the dorsal part of the LC complex, but also on presumed cholinergic and cholino-sensitive neurons (cf. Pompeiano et al., 1991), which are mostly located more ventrally in the peri-LCα and the neighbouring dorsal PRF. This possibility would be of great functional significance, in view of the tonic inhibitory influences that the LC neurons exert on the dorsal PRF (cf. Pompeiano et al., 1991).

The hypothesis that VP acts on noradrenergic LC neurons as an excitatory neurotransmitter or neuromodulator was originally supported by results of biochemical studies showing that intracerebroventricular administration of VP increased, and that of antivasopressin serum decreased, NA turnover in a number of structures including the LC (Tanaka et al., 1977a,b; cf. Schwartzberg et al., 1981). More direct evidence was provided by Olpe and Baltzer (1981) who found that microiontophoretic administration of (Arg^8)- and (Lys^8)-VP to rats anaesthetized with chloral hydrate increased the firing rate in most (87.1%) noradrenergic LC neurons, characterized by their slow and regular firing pattern and their typical responses to a peripheral painful stimulus (cf. Foote et al., 1983). An additional observation, was that a smaller proportion of non-noradrenergic neurons in the area of LC (34.3%) also had an excitatory response to local administration of (Lys^8)-VP. This indicates that, under identical conditions, the specificity of this peptide to elicit cell excitation is not so strict as originally thought, in that it acts not only on noradrenergic, but also to a lesser extent on non-noradrenergic neurons.

The conclusion reported above is supported by the results of our experiments showing that VP may modulate posture and VS reflexes by acting not only on the LC complex, but also on the peri-LCα and the dorsal PRF. However, due to the interaction between the two populations of neurons (cf. Pompeiano et al., 1991), the induced changes in posture as well as in gain of the VS reflexes following injection of VP into the peri-LCα and the neighbouring dorsal PRF, where cholinergic neurons predominate, were opposite in sign to those obtained after injection of the same neuropeptide into the LC complex, where noradrenergic neurons are located. These effects depended on a specific action of the neuropeptide, because injection of one equal volume of the vehicle alone was ineffective. Moreover, the close localization of the pontine structures giving rise to the reciprocal changes in posture and reflexes described above excludes the possibility that these effects result from local effects of VP on blood vessels (cf. Van Zwieten et al., 1988).

In our experiments (Andre et al., 1992a), microinjection of small doses of VP in the LC complex of one side produced postural asymmetry, characterized by an increase of the extensor rigidity in the ipsilateral limbs and a slight decrease or no change in postural activity in the contralateral limbs. These postural changes were also associated with a decrease in amplitude of modulation and thus in the response gain of the extensor triceps brachii of both sides to animal tilt (see Figs. 1 and 2). This effect was also associated with slight changes in the phase angle of the responses.

The increase in postural activity in the ipsilateral limbs was due to VP-induced discharge of noradrenergic CS neurons (cf. Holstege and Kuypers, 1987). These neurons activate ipsilateral limb

extensor (and flexor) motoneurons by exerting a direct excitatory influence on them (cf. Fung et al., 1991) as well as an inhibitory influence on the corresponding Renshaw cells (Fung et al., 1987, 1988; cf. Fung et al., 1991). In addition, the noradrenergic and NA-sensitive LC neurons (cf. Foote et al., 1983) exert an inhibitory influence on dorsal pontine structures such as the peri-LCα and the adjacent dorsal PRF (cf. Pompeiano et al., 1991), whose cholinergic and/or cholino-sensitive neurons, however, activate through a direct tegmento-reticular projection (Sakai et al., 1979; Jones and Yang, 1985) the inhibitory area of the medullary reticular formation (Lai and Siegel, 1988; see Pompeiano et al., 1991 for references). This area may actually inhibit spinal motoneurons by activating, partly at least, the corresponding Renshaw cells (cf. Pompeiano, 1984). Thus, the increase in postural activity which occurs following VP activation of LC complex neurons results from not only an increased discharge of facilitatory CS neurons, but also from a reduced discharge of dorsal pRF neurons and the related medullary inhibitory RS neurons.

The mechanism by which VP activation of noradrenergic LC neurons reduces the response gain of the forelimb extensor to sinusoidal stimulation of labyrinth receptors should also be considered. Experiments with unit recording have shown that the increased activity of limb extensor motoneurons which occurs during side-down tilt of the animal (cf. Manzoni et al., 1983a) is associated with not only an increased discharge of excitatory VS neurons (cf. Boyle and Pompeiano, 1980; Schor and Miller, 1982; Marchand et al., 1987), but also with a reduced discharge of both excitatory CS neurons (Pompeiano et al., 1990) and inhibitory RS neurons (Manzoni et al., 1983b). The opposite result would follow animal tilt in the other direction.

The role that the CS neurons as well as the RS neurons have during the VS reflexes may depend on the predominant activity, in the animal at rest, of either the CS or the dorsal pRF neurons and the related medullary inhibitory RS neurons (cf. Pompeiano et al., 1991). When the discharge of the LC, and thus of the CS neurons, predominates over that of the PRF neurons and the related inhibitory RS neurons, the higher the firing rate of the CS neurons in the animal at rest the greater the disfacilitation which affects the extensor motoneurons during side-down animal tilt. These motoneurons would then respond less efficiently to the excitatory VS volleys elicited for the same direction of animal orientation, leading to a reduced gain of the responses of forelimb extensor muscles to labyrinth stimulation. On the other hand, when the activity of the PRF neurons and the related medullary inhibitory RS neurons predominates over that of the LC and the CS neurons, the higher the firing rate of these RS neurons in the animal at rest, the greater the disinhibition which affects the extensor motoneurons during side-down animal tilt. These motoneurons would then respond more efficiently to the same excitatory VS volleys elicited by given parameters of stimulation, leading to an increased response gain of forelimb extensor muscles to labyrinth stimulation.

In decerebrate cats, the discharge rate of the LC neurons in the animal at rest is higher than that observed in intact animals, due to interruption of supra-mesencephalic descending pathways exerting inhibitory influences on the LC neurons (cf. Pompeiano and Hoshino, 1976; Pompeiano et al., 1990), while the activity of the dorsal PRF neurons (Pompeiano and Hoshino, 1976) and the related medullary inhibitory RS neurons (Manzoni et al., 1983b) is depressed. In these instances, EMG modulation of the limb extensors to labyrinth stimulation is of small amplitude (Manzoni et al., 1983a). Tonic activation of the LC neurons following local injection of VP would further reduce this EMG modulation, partly by utilizing the CS system, and partly by suppressing the tonic activity of the dorsal PRF neurons and the related medullary inhibitory RS neurons.

It is of interest that the LC of one side has under its tonic inhibitory control not only the PRF of the ipsilateral but also, to a lesser extent, that of the contralateral side (cf. Pompeiano et al., 1987). This could explain why activation by VP of the LC of one side reduced the response gain to labyrinth stimulation not only of the ipsilateral, but also of the contralateral triceps brachii.

This interpretation of our findings is supported by results of previous experiments showing that postural and reflex effects opposite in sign to

those induced by local injection into the LC of VP were obtained either from electrolytic lesioning of the LC of one side (D'Ascanio et al., 1985) or from local injection into the structure of the α_2-adrenergic agonist, clonidine (Pompeiano et al., 1987). This led to functional inactivation of the noradrenergic and NA-sensitive LC neurons, thus releasing the activity of the PRF neurons and the related medullary inhibitory RS neurons from inhibition.

In addition to the findings reported above, our experiments have shown that VP may modulate posture and VS reflexes by acting not only on the LC complex (Andre et al., 1992a), but also on the peri-LCα and the dorsal PRF (Andre et al., 1992b). In particular, unilateral injection of small doses of VP in the dorsal part of the pontine tegmentum reduced the postural activity in the ipsilateral limb extensor, while the amplitude of the EMG responses of the corresponding triceps brachii to animal tilt, and thus the response gain of this muscle to labyrinth stimulation, were increased (see Fig. 3). These findings can be understood if we consider that the neuropeptide exerts a tonic excitatory influence on the ipsilateral dorsal PRF neurons. The increased discharge of these neurons would then activate the related medullary inhibitory RS neurons, leading to reduced postural activity in the ipsilateral limbs (cf. Pompeiano et al., 1991). On the other hand, since the presumed inhibitory RS neurons show a response pattern to animal tilt (β-responses; Manzoni et al., 1983b) opposite in sign to that displayed by the excitatory VS neurons (α-responses; Boyle and Pompeiano, 1980; Schor and Miller, 1982; Marchand et al., 1987), it is likely that the increased discharge of the medullary RS neurons, which probably occurs in the animal at rest after VP activation of the PRF neurons, would lead to an increased disinhibition which affects the ipsilateral extensor motoneurons during side-down tilt. These motoneurons would then respond more efficiently to the same excitatory VS volleys elicited by given parameters of labyrinth stimulation, leading to increased gain of the EMG responses of forelimb extensor muscles to animal tilt. These responses were always of the α-type, as in the control situation (Schor and Miller, 1981; Manzoni et al., 1983a; Ezure and Wilson, 1984). They were thus characterized by increased EMG activity during ipsilateral tilt and decreased activity during contralateral tilt. This interpretation of our findings is supported by the results of previous experiments showing that, in decerebrate cats, a decreased postural activity associated with an increased gain of the VS reflexes could also be elicited by local injection in the ipsilateral PRF of either the cholinergic agonists, carbachol and bethanechol, leading to direct activation of presumed cholinergic and cholino-sensitive neurons (Barnes et al., 1987), or of the β-adrenergic antagonist, propranolol, suppressing the inhibitory influence of the noradrenergic LC neurons on the related dorsal PRF neurons (D'Ascanio et al., 1989a). These findings indicate that the dorsal pontine reticular structures involved in the vasopressinergic control of posture and the VS reflexes are cholino-sensitive as well as NA-sensitive.

In addition to ipsilateral effects, local injection of VP in the peri-LCα and the dorsal PRF also had contralateral effects due to involvement of either the PRF or the LC complex of the opposite side (Sakai et al., 1977; Jones and Yang, 1985). In a first group of experiments in which VP injection into the PRF of one side decreased postural activity of the ipsilateral, but also to a lesser extent, of the contralateral triceps, the response gain of the ipsilateral and, less, of the contralateral triceps brachii to animal tilt increased slightly (see Fig. 3). Moreover, the phase angle of the responses, which remained of the α-type, was not greatly modified. These effects were attributed to VP activation of both the ipsilateral and the contralateral PRF. In a second group of experiments, however, the changes in posture as well as those in the response characteristics of the forelimb extensors to labyrinth stimulation induced by VP injection into the PRF of one side behaved as though this structure activated mainly the LC complex of the opposite side. In these instances, tonic activation of the excitatory LC neurons could account for the increased postural activity in the contralateral limbs after unilateral injection of VP into the PRF. The same projection could also account for the reversal of the response pattern of the contralateral limb extensor to animal tilt (see Fig. 4A,B). On this side, the reduced discharge of the excitatory CS neurons

which occurs during side-down tilt (β-responses; Pompeiano et al., 1990), leading to disfacilitation of the corresponding extensor motoneurons, would counteract the excitatory influence that the VS neurons exert on the corresponding limb extensor motoneurons for the same direction of animal displacement (cf. Marchand et al., 1987), leading to reversal of the response pattern of the limb extensor from the α- to the β-type. It is of interest that reversal of the response pattern of the contralateral limb extensor motoneurons to animal tilt, similar to that described above, was observed in previous experiments after unilateral injection in the dorsal PRF of a β-adrenergic antagonist (D'Ascanio et al., 1989a), which released the activity of the PRF neurons from inhibition.

The effect of VP injection on the contralateral limb extensor in these latter two groups of experiments depended on the location of the injection sites within the dorsolateral and the dorsomedial part of the pontine tegmentum, respectively.

We cannot conclude our discussion without mentioning that, in some experiments (reported in Andre et al., 1992b), the postural changes produced by the VP injection into the dorsal PRF of one side, described above, were interrupted from time to time by transient episodes of postural atonia, which lasted several minutes and affected mainly the ipsilateral limbs. These episodes were similar to those obtained after unilateral injection into the peri-LCα and the adjacent dorsal PRF either of the cholinergic against, carbachol (Barnes et al., 1987; D'Ascanio et al., 1988; cf. also Katayama et al., 1984; Vanni-Mercier et al., 1989), or of the β-adrenergic antagonist, propranolol (D'Ascanio et al., 1989a), which led to strong activation or disinhibition of the corresponding dorsal pontine reticular neurons, respectively. In these instances, the activity of the dorsal PRF neurons and of the related medullary RS neurons was probably so prominent as to produce intense postsynaptic inhibition of extensor α-motoneurons, as seen after local administration, in the same dorsal PRF region, of cholinergic agonists (Morales et al., 1987), suppressing the EMG responses of the corresponding triceps brachii to animal tilt (at 0.15 Hz, $\pm 10°$).

Concluding remarks

In conclusion, it appears that the vasopressinergic afferents to the LC complex and the dorsal PRF exert a modulatory influence on posture as well as on the gain of the VS reflex through an appropriate circuit. This circuit is made up of presumed noradrenergic inhibitory and cholinergic excitatory regions (see Pompeiano et al., 1991; Tononi and Pompeiano, 1995). In particular, there is evidence that noradrenergic and NA-sensitive LC neurons are inhibitory on the dorsal PRF neurons. On the other hand, the cholinergic and cholino-sensitive PRF neurons are excitatory on medullary inhibitory RS neurons. The increased discharge of LC neurons following local injection of VP into the LC complex, which involves not only excitatory CS neurons, but also LC neurons exerting an inhibitory influence on the dorsal PRF, increases postural activity in the ipsilateral limbs, while reducing the gain of the corresponding VS reflexes. On the other hand, the increased discharge of the dorsal PRF neurons and of the related medullary inhibitory RS neurons following local injection of VP into the dorsal PRF decreases postural activity in the ipsilateral limbs, but greatly enhances the amplitude of EMG modulation and thus the response gain of the ipsilateral limb extensors to labyrinth stimulation.

The neuronal circuit made by the inhibitory LC neurons and the excitatory PRF neurons may thus operate as a *variable gain regulator* acting on motoneurons during the VS reflex. Since both these populations of neurons are not only VP-sensitive but also NA-sensitive, we postulate that one of the possible roles of the vasopressinergic excitatory input to both the LC and the dorsal PRF is to antagonize the noradrenergic inhibitory influence that the LC neurons exert both on themselves through α_2-adrenoceptors (see Pompeiano et al., 1987) and on the dorsal pontine reticular neurons through β-adrenoceptors (see D'Ascanio et al., 1989a). It is of interest that both the LC complex and the dorsal PRF intervene in the regulation of posture and possibly also of the gain of VS reflexes during the sleep-waking cycle (cf. Tononi and Pompeiano (1995) for references). This finding raises the question of the possible modulatory role that the vaso-

pressinergic system originating from hypothalamic nuclei and projecting on the same pontine tegmental structures exert in the static and dynamic control of posture during the different animal states.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 5.11

The vasopressin deficient Brattleboro rats: A natural knockout model used in the search for CNS effects of vasopressin

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Behavioral neuroscience is using more and more gene knockout techniques to produce animals with a specific deletion. These studies have their precedent in nature. A mutation may result in a limited genetic defect, as seen in the vasopressin (VP) deficiency in the Brattleboro rat. The mutation is in a single pair of autosomal loci, and the sequences of VP gene from wild-type and homozygous Brattleboro rats are identical except for a single nucleotide deletion in the second exon. The deletion results in the synthesis of an altered VP precursor that is unable to enter the secretory pathway. The genetic disturbance results in a central diabetes insipidus comparable to that found in humans. Starting with our work during the early 1970s we found that the genetic defect in the availability of VP causes deficits in central nervous system (CNS) functions. Behavioral processes from cognition to drug tolerance appeared to be disturbed by the absence of VP, but not all behaviors are affected. The specificity of the absence of VP in causing behavioral deficits is shown in many cases. However, certain deficits are due to genetic factors other than the deletion of the VP gene. The picture is further complicated by differences in testing conditions, the absence of proper controls, i.e. heterozygous and wild-type Brattleboro rats, sex, compensation phenomena, and the absence of neuropeptides co-localized with VP. Interestingly, an age dependent spontaneous shunt to a heterozygous phenotype in vasopressinergic neurons might also compensate for the disturbance. Accordingly, findings in knockout animals should be interpreted with caution. One should realize that brain functions are modulated by multiple neuropeptides and that neuropeptides possess multiple CNS effects.

Introduction

Nowadays, an essential strategy to delineate the function of a particular gene product and the physiological events in which it participates, is the inactivation of the gene in the genome of an organism. Homologous recombination of genes in mouse embryonic stam cells has been the basis of the creation of numerous 'knockout' mice during the last number of years (e.g. Cambell and Gold, 1996). The mice have provided clues to the function of many proteins in particular in developmental processes. More recently knockout mice have been created to track functions of proteins in the adult nervous system, in which behavioral responses were the prime focus. Such knockouts concern regulators of intraneuronal cascades, e.g. ion channels, receptors, neurotrophic factors, intracellular messengers as well as neuropeptides, plasticity and behavior (Shastry, 1995; Wehner et al., 1996; Chen and Tonegawa, 1997; Nogues, 1997; Silva et al., 1997; Young et al., 1997). Sometimes, these knockouts have surprised researchers because of unexpected phenotypes or lack of an expected phenotype. Often, the precise physiological function in the adult brain is difficult to extract due to interference and compensation during development, and due to the genetic background of the animals used. In the field of neuropeptides, the first animal that missed only one functional gene was the Brattleboro rat.

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In 1964, Valtin and Schroeder reported on the selection of a strain of rats with familial hypothalamic diabetes insipidus which was designated as the Brattleboro strain. The disease manifests itself in polyuria and polydypsia. These rats were derived from a Long–Evans strain of the Rockland colony. The homozygous mutants (−/−) lack the ability to synthesize vasopressin (VP) due to a mutation in a single pair of autosomal loci (Valtin and Schroeder, 1964). The heterozygous littermates (+/−) have a partial deficit in the synthesis (Valtin et al., 1965) and release of VP (Moses and Miller, 1970; Miller and Moses, 1971). Schmale and Richter (1984) elucidated the genetic defect after isolating the mutant gene. They found that the sequences of the VP genes from normal wild-type (+/+) homozygous Brattleboro rats are identical except for a single nucleotide deletion in the second exon. Although the coding for the VP part is normal, the deletion results in the synthesis of an altered VP precursor, which is unable to enter the secretory pathway.

It was the first animal with a genetic defect causing a disease similar to that seen in humans. Heinz Valtin suggested to one of us to use this animal to further investigate the role of VP on learning and memory processes. As he wrote in a commentary on his first paper (Valtin and Schroeder, 1964) in 'Milestones in Nephrology' in 1997 '...it was not until some 34 years later when Mark Knepper referred to the Brattleboro rat as the first 'knockout animal''. We believe that we were the first to use a knockout model for the study of behavior. Over the last 30 years a wealth of experimental findings and experience have been accomplished on this animal model. Focusing on the central nervous system (CNS) effects of VP we have learned about its role in learning and memory processes, but we also experienced pitfalls. Our experience may be prototype of the current problems that one may encounter when knockout animals are used to delineate central functions of neuropeptides. Accordingly, the aim of this chapter is to summarize the recent knowledge on the behavioral and other brain functions in the Brattleboro rats with hereditary diabetes insipidus with special emphasis on our own experience.

Behavioral studies: Learning and memory

In our first study we found that the memory function of homozygous diabetes insipidus (HoDi) male Brattleboros was impaired as compared to Brattleboro heterozygous (HeDi) and normal Wistar control animals. It was first assessed in a one-trial learning passive avoidance test which is since long the most frequently used paradigm to study consolidation and retrieval of memory in rodents (McGaugh, 1966). Performance was measured 24 h after the learning trial (De Wied et al., 1975). Arginine-8-vasopressin (VP) or des-glycinamide-9-lysine-8-vasopressin (DGLVP), given immediately after the learning trial, restored the behavior of the HoDi rats. The fact that the endocrinologically inactive VP analog DGLVP was also effective suggested that the beneficial effect of VP could not be ascribed to the restoration of water metabolism. Since the HoDi rats showed avoidance behavior comparable to that of HeDi litter mates when tested immediately after the learning trial, but not if investigated 24 h later, it was concluded that memory rather than learning processes are disturbed in the absence of VP. Bailey and Weiss (1979) also found that passive avoidance behavior of female HoDi rats was inferior to that of heterozygous controls but the animals were not amnesic for the task. However, subsequent studies failed to replicate equivocally these studies. Miller (1982) found that the behavior of HoDi rats trained with various shock intensities was not different from that of Long–Evans rats. He also found that chronic treatment of Di rats with pitressin tannate in oil, a pituitary extract with high ADH-activity, suppressed passive avoidance behavior. This was opposite of what was expected: we reported that pitressin facilitates retention of shuttle-box avoidance behavior in posterior lobectomized rats (De Wied, 1969) and Wistar rats (De Wied and Bohus, 1966). Ambrogi Lorenzini et al. (1988) also reported that passive avoidance behavior was equally well acquired in HoDi and HeDi rats and retained even better by the HoDi animals. These authors however used rather high shock intensities. Van Haaren et al. (1985) found that passive avoidance behavior was impaired in HoDi as compared to HeDi rats at 35 days of age but not at 60 and 120 days. The

authors explained the contradictory results reported in the different studies as a consequence of age differences of the Brattleboro rats used. Indeed, this may well be an important factor since Van Leeuwen et al. (1989) and Van Leeuwen (1992) detected a small number of hypothalamic neurons in the homozygous mutants, which underwent a switch to a heterozygous phenotype. These increased in a linear fashion from 0.1% to 3% of the VP cells as a function of age. In the meantime the original experiment by De Wied et al. (1975)have been repeatedly replicated by us using 80–100 day old male Brattleboro rats of the Dutch TNO Zeist line. In one study the wild-type +/+ littermates were used with the −/− and +/− mutants, whereas another experiment used a parent Long–Evans strain as the control (Bohus, 1979; Bohus and De Wied, unpublished results). In the third replication homozygous and heterozygous males were used (Borrell et al., 1985; Bohus et al., 1993). Finally, in the fourth replication we used again the homozygous, heterozygous and wild-type Brattleboros (Drago and Bohus, 1986). All these replications showed that long-term memory, i.e. retention of the passive avoidance 24 h or later after learning, is seriously disturbed in the homozygous DI mutants in comparison to heterozygous and wild-type littermates. As in the first study (De Wied et al., 1975), treatment with VP and DGLVP normalized the behavior of DI rats (see Fig. 1). Unfortunately, data from older Brattleboro rats in this test are not available.

In multiple trial learning paradigms such as the shuttle-box or pole-jumping active avoidance tests, Bohus et al. (1975), found that acquisition of conditioned avoidance response in HoDi rats was nearly normal but the behavior was extinguished more rapidly than that of HeDi or Wistar rats. Celestian et al. (1975) reported that HoDi rats were inferior in acquiring shuttle-box avoidance behavior, since only 30% of the animals reached the learning criterion. However, those rats that achieved the criterion maintained the response better than the control animals. Miller et al. (1976) found that rats homozygous for diabetes insipidus were more deficient in escape, and avoidance responding than were their

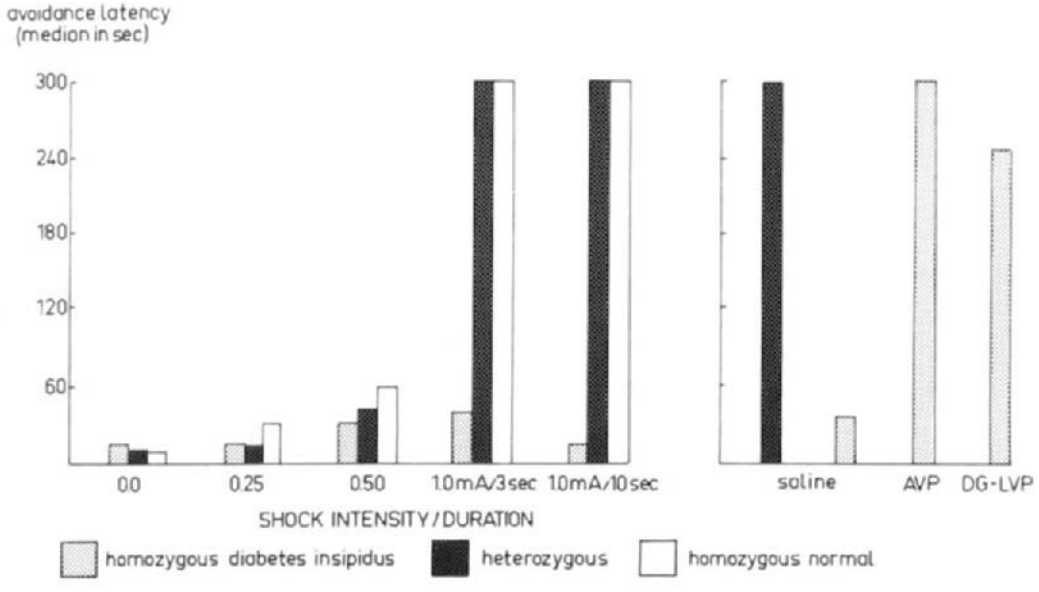

Fig. 1. Retention of a one-trial learning, step-through passive avoidance response in Brattleboro rats as the function of shock intensity at the learning trial. The left panel shows the avoidance latencies of the −/− (homozygous diabetes insipidus), +/− (heterozygous), and +/+ (wild-type homozygous normal) males at the 24 h retention test. The right panel displays the influence of s.c. VP (arginine-vasopressin) and DGLVP (desglycinamide-8-lysine-vasopressin) treatment (1 μg/rat) given immediately after learning on the passive avoidance behavior (Bohus, 1979; Bohus and De Wied, unpublished data).

heterozygous or normal controls. Although the heterozygous rats improved their escape behavior when the shock intensity was increased, their avoidance behavior was not improved. Surprisingly, analysis of variance of the data eliminated the differences in behavior between the three groups of rats. In a subsequent study Miller (1982) reported that acquisition of active avoidance behavior was even superior in the Di rat.

We have also replicated the active avoidance studies in a shuttle-box when the wild-type littermates became available (Bohus, 1979; Bohus and De Wied, unpublished data). The results of this study are shown in Fig. 2. The findings again suggested that the homozygous mutants are primarily impaired in the extinction of the response. They are practically unable to retain the response in comparison to the slightly disturbed heterozygous and non-disturbed wild-type littermates. Remarkably, the parent Long–Evans strain of a French origin was practically unable to master the shuttle-box avoidance task, and showed highly impaired extinction behavior (data not shown). Finally, in a one-session mass-trial acquisition paradigm the shuttle-box avoidance behavior of the three genotypes was investigated (Drago and Bohus, 1986). In this situation the acquisition behavior of the HoDi males was impaired in comparison to the HeDi and wild-type Brattleboros. We observed also a difference between the heterozygous and wild-type rats: the latter were more superior in acquiring the behavior than the former. Using other paradigms did not equivocally indicate disturbances in learning and memory processes. Brito et al. (1981) showed that HoDi rats adapted more slowly than Long–Evans rats in a T-maze and made fewer correct responses when learning a visual and olfactory discrimination task, but the animals were not inferior in avoidance tasks. These authors suggested that not all aspects of learning and memory are equally affected in the HoDi rat. In a subsequent study (Brito et al., 1982; Brito, 1983) HoDi rats appeared to be impaired in temperament-related tasks, i.e. time-spent-eating, time-to-emerge, and adaptation to the T-maze and the runway. HoDi rats performed worth relative to control Long–Evans rats in a task involving working memory, i.e. T-maze alternation and tasks involving reference memory, i.e. visual and olfactory discrimination. HoDi rats in their hands showed as good a memory as that of controls on shock motivated tasks, i.e. approach-avoidance conflict and step-through passive avoidance. HoDi rats were also performing as well in a task involving

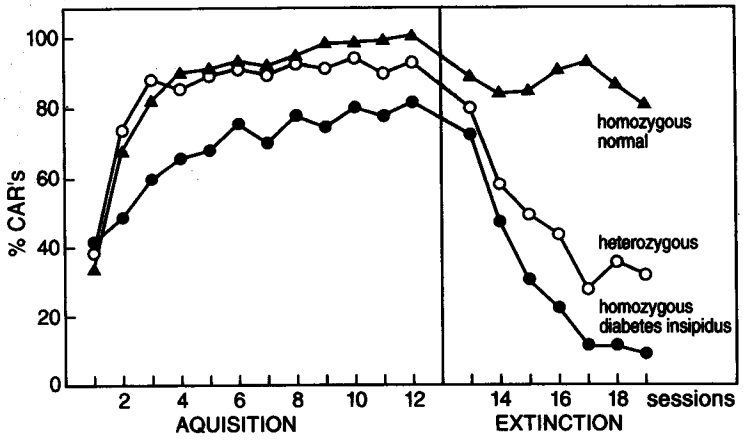

Fig. 2. Acquisition and extinction of two-way shuttle-box avoidance in male Brattleboro rats. The rats were subjected to 10 conditioning trials per day with variable intertrial interval averaging 60 s. The duration of the conditioned stimulus was 5 s followed by an unconditioned stimulus of electric foot shock of 0.5 mA a.c. The number of avoidance responses served as the measure of conditioned behavior. For further explanations see Fig. 1 (Bohus, 1979; Bohus and De Wied, unpublished data).

memory for a species specific behavioral response as defensive burying and learned a one-way active avoidance task at least as well as the control animals. They suggested that HoDi rats have altered temperamental dispositions, enhanced neophobia, and timidity. They commented on the possibility that the difference in genetic background may be a more critical determinant of behavioral disturbances in HoDi rats than VP deficiency per se. Carey and Miller (1982) who were unable to find differences in active and passive avoidance behavior in HoDi rats as compared to control animals also suggested this.

Social recognition is used as another measure to study memory processes in relation to VP (Dantzer and Bluthë, 1993). It is based on the tendency of rodents to inspect familiar conspecifics less than unfamiliar ones. Social recognition is impaired in HoDi Brattleboro rats as compared to Long–Evans animals in the same way as in control rats following microinjection of a V1 VP receptor antagonist in the mediolateral septal area (Engelmann and Landgraf, 1994).

We were the first to find that open field behavior did not decline in subsequent sessions in both HoDi and HeDi rats. Accordingly, in contrast to Wistar animals, no habituation occurred in the Brattleboro rats (Bohus et al., 1975). Warren and Gash (1983) also found hyperresponsiveness to shock and acoustic stimuli in the VP-deficient rat. Williams et al. (1983b) reported that 5 μg VP given s.c., which is a high dose, depressed both open field behavior and photochamber activity but it had less effect on normal Long–Evans rats. A dose of 1 μg was much less active. In terms of learned behavior HoDi and control rats displayed similar within-session habituation when comparisons were made following the same treatment conditions.

Laycock and Gartside (1985) found that the performance of HoDi rats in a Skinner box to a fixed ratio was better in animals, which were given daily injections of pitressin tannate. Once the initial task was learned, the authors did not find marked differences in discrimination learning between control and treated Brattleboro or Long–Evans rats. Brito (1985) studied a progressive-ratio schedule operant behavior and quinine-induced drinking suppression of the Brattleboro rats. The HoDi mutants responded less than Long–Evans animals but they suppressed drinking as well as the Long–Evans rats. In a conditioned freezing paradigm, HoDi rats showed less freezing then the HeDi animals (Ambrogi Lorenzini et al., 1988). Stoehr et al. (1993) also found that HoDi rats displayed attenuated conditioned freezing behavior in a contextual learning paradigm when compared to HeDi and Long–Evans rats. Animals were given a series of 3 foot shocks daily for 4 consecutive days and the next day placed in the chamber where they had received the foot shocks. Thus, the HoDi rat may not remember an aversive experience as well as the HeDi and Long–Evans controls.

Gaffori et al. (1985), compared the behavior of HoDi rats with that of HeDi, the wild-type normal and Wistar animals in various paradigms. HoDi rats exhibited a higher rate of ambulation, rearing, grooming, sniffing, and defecation in a small and a large open field but a lower activity in the middle of the large open field. Ten years earlier Bohus et al. (1975) had found that the Brattleboro strain had a higher rearing activity in the middle of the large open field, no differences in grooming, and a slightly lower defecation. Accordingly, the behavioral profile of the Brattleboro rats had somewhat changed during the years between the first experiments and these last ones. Genetic drift is well known as the result of inbreeding. This is an additional factor to the breeding difficulties of the Brattleboros (Valtin, 1967). HoDi rats also habituated more slowly than HeDi, wild-type and Wistar rats (Gaffori et al., 1985), while in the study of Bohus et al. (1975) habituation was slow in HeDi rats as well. When a food motivated visual discrimination task was used, HoDi and HeDi rats made fewer correct responses. The same was found in a hole-board food-searching task. HoDi and HeDi rats had a somewhat reduced learning, working, and reference memory in this task when compared to the wild-type and Wistar animals. No difference was found between the various groups in learning and reversal learning of an aversively motivated T-maze task. However, HoDi rats made more errors in the retention tests. In both HoDi and HeDi rats passive avoidance behavior was equally impaired, but HoDi rats were more impaired in acquisition

and extinction of pole-jumping avoidance behavior than HeDi rats which performed less than +/+ and Wistar rats. These studies confirmed our original studies in principle and showed that aversively as well as appetively motivated behavior is disturbed in rats with a deficient VP system, but that not all behaviors are equally affected. If disturbances were found, they could be restored by the administration of DGAVP.

In one of the most recent studies with the Brattleboro rats Brot et al. (1992) studied the sexual dimorphism in the extinction of a conditioned taste aversion behavior. Male wild-type Long–Evans rats showed less rapid extinction of LiCl induced conditioned taste aversion of a sucrose solution than females of this genotype. This sex difference is absent in the homozygous and heterozygous Brattleboros. The authors suggest that intact VP levels are necessary to express this sexually dimorphic behavior. The significance of VP as a messenger of sex hormone dependent behavioral expression is supported by neurobiological evidence. Part of the extrahypothalamic vasopressinergic system is sexually dimorphic and depends on testosterone. This dimorphism is primarily present in the vasopressinergic neuronal system originating from the bed nucleus of the stria terminalis (e.g. Van Leeuwen et al., 1985; Miller et al., 1989a,b).

Studies in different genotypes

The discrepancies in the results of the various learning and memory studies have never been adequately explained. One explanation given has been based on differences in genotypes. The differences in behavior might therefore be more related to the genetic background of the animals than the absence of VP. Several authors decided to explore a possible genotype influence by using different strains and sources of animals.

Herman et al. (1986a) studied the behavior of Brattleboro rats obtained from two different sources, one strain bred at Rochester, NY, USA (RO/DI) and one from the colony bred at Charing Cross Hospital, London, UK (CC/DI). This latter line was the original source we introduced in Holland. These Brattleboros were compared to normal Long–Evans rats bred in Rochester and London, respectively. Both strains of DI rats acquired goal-approach behavior in a straight runway at similar rates. Following shock in the runway goal box, RO/DI rats showed marked recovery of running behavior relative to CC/DI rats, indicating that RO/DI rats did not remember the aversive experience as well as CC/Di animals. All DI rats showed reductions in goal-approach speed on the first post-shock trial, indicating that the aversive experience is learned. The CC Long–Evans rats acquired goal-approach behavior more slowly than the RO Long–Evans animals, but neither Long–Evans animals showed substantial recovery of goal-approach behavior after shock: they did remember the aversive experience. CC/DI rats showed impaired acquisition of a delayed non-match to sample task relative to RO/DI animals. All groups demonstrated the ability to utilize representational memory to solve the delayed non-match to sample problem once the contingency was learned. The results indicate that DI and normal Long–Evans rats from different colonies indeed show marked differences in behavior. However, learning and memory disturbances were found in DI rats of both lines. Herman et al. (1986b) also studied the behavior of the Roman High Avoidance rat homozygous for diabetes insipidus (RHA:di/di). The RHA:di/di rat was bred to be congenic with the parent normal Roman High Avoidance (RHA:+/+) strain, differing from it only by the genes coding for diabetes insipidus. According to the authors, this was a useful model to study the behavioral effects of VP deficiency because genetic variables unrelated to VP-deficiency may confound the interpretation of data. Animals were tested in an open field, and on tests of approach-avoidance, spatial memory, and passive avoidance. The RHA:di/di rats displayed retarded habituation and increased rearing, defecation and ambulation in the central area of the open field. The RHA:di/di rats showed substantial recovery of goal-approach after shock whereas RHA:+/+ did not, indicating a memory disturbance in the VP-deficient rat. Both strains were able to solve a delayed non-match to sample task but RHA:di/di showed a slower acquisition of the contingency and significant faster run times of choice trials of

the paired run procedure. No difference was found between the strains in passive avoidance behavior. The authors interpreted these results as effects on arousal and attention which secondary affect performance of memory-related tasks. The DI gene of the Brattleboro rat was also introduced by Colombo et al. (1992) into the M520 strain of rats to produce a new strain of genetically VP-deficient rats. These rats differed from HeDi and wild-type M520 rats in terms of daily water intake, delayed acquisition of a food-rewarded alternation task in a T-maze and an impaired ability to retain spatial information. The behavioral profile of these rats is therefore more or less similar to that of the HoDi Brattleboro rat in our studies. The HeDi of the M520 strain exhibited an intermediate performance but their water intake was normal. In contrast to that of HoDi Brattleboro rats, habituation to a novel environment of HoDi M520 rats was somewhat faster than that of controls. These authors concluded that adaptation to a novel environment might not be under the control of the Di gene.

Other hormones and avoidance behavior

Although the primary defect of the Brattleboro rat is the disturbance in the synthesis of VP, other endocrine abnormalities were found (see later in this chapter). This may be important since many neuropeptides and peripheral hormones modulate learning and memory processes (Bohus, 1994; Kovacs and De Wied, 1994). For example Drago and Bohus (1986) found that hyperprolactinaemia induced by homografting two adenohypophyses under the kidney capsule alleviated a number of the behavioral alterations of HoDi rats but it did not affect water metabolism.

In a single session shuttle-box avoidance learning paradigm, hyperprolactinaemic HoDi rats acquired the conditioned response as well as non-hyperprolactinaemic HeDi and wild-type littermates did. Pituitary implantation and consequent hyperprolactinaemia also alleviated passive avoidance retention deficit in the HoDi rats. Hyperprolactinaemia reduced sensitivity to electric foot shock and facilitated the acquisition of a pole-jumping avoidance response in all Brattleboro genotypes, but failed to affect the passive avoidance behavior of HeDi and wild-type rats. Accordingly, prolactin may be one candidate to compensate for certain CNS deficits of the HoDi rat, and in this way possess selective behavioral effects on certain genotypes of the Brattleboro strain. The neuropeptides ACTH 4–10 and met-enkephalin also affect the behavioral response of the HoDi rat in a passive avoidance test. Met-enkephalin resembles VP and DGLVP but is less effective. The interesting aspect of the action of ACTH 4–10 given immediately after a learning session is genotype specific. It normalizes memory of the HoDi rats, but not that of the He littermates (Bohus, 1979; Bohus et al., 1993).

A unique relationship exists in behavioral terms between peripherally circulating epinephrine and the central VPergic system. Epinephrine originating from the adrenal medulla is a powerful modulator of the consolidation of memory (McGaugh, 1989). We reported that epinephrine is unable to correct the memory deficit of HoDi rats, whereas the hormone is active in the HeDi genotypes. Conversely, VP administration fails to affect the memory of both HoDi and HeDi Brattleboros when the source of the circulating E, i.e. the adrenal medulla, is removed (Borrell et al., 1985; Bohus et al., 1993). Accordingly, VP and epinephrine mutually cooperate in modulating memory processes.

Drug tolerance

The view that some aspects of tolerance development may be analogous to learning and memory processes stimulated the study on the influence of neurohypophyseal hormones on opioid and ethanol tolerance. Krivoy et al. (1974) reported that DGLVP facilitated the development of resistance to the antinociceptive action of morphine. Tolerance to morphine-induced behavioral changes in freely moving rats was accelerated following an i.c.v. injection of DGLVP (Cools et al., 1977). HoDi rats showed impaired development of tolerance to morphine analgesia, a deficit that could be restored by treatment with VP or DGAVP (De Wied and Gispen, 1976). Brattleboro rats also showed a deficient onset and persistence of cocaine-induced behavioral sensitization (Post et al., 1982). Deficits in tolerance to ethanol in Brat-

tleboro rats have also been reported (Pittman et al., 1982). The rats failed to develop tolerance to ethanol as measured by a time-dependent fall in body temperature. The HoDi and HeDi rats also exhibited an increased sensitivity to the hypothermic effects of ethanol, when compared to Sprague–Dawley rats. This finding agreed with other studies which showed that VP and related fragments facilitated the development of tolerance to the hypothermic and sedative effects of alcohol (Hoffman et al., 1979; Rigter et al., 1980; Hoffman, 1994).

Other behavioral effects

An increased startle response to electric tail shock was found by Gash et al. (1982) indicating that the Brattleboro rat is hyperresponsive to electric shock. This could not be corrected by treating the animals s.c. with VP. Also jump threshold to electric shock was lower in Brattleboro rats (Bodnar et al., 1982). This was first reported by Bohus et al. (1975), but HeDi rats were also more sensitive than Wistar controls. VP has analgesic properties (Berntson and Berson, 1980; Bodnar et al., 1980). The Brattleboro rat exhibits hyperalgesia and an impaired analgesic response to stress. These deficits could be reinstated either fully or partially by VP. The analgesic response to morphine however was similar in Brattleboro and normal rats (Bodnar et al., 1980, 1982).

Motor disturbances in HoDi rats following a first i.v. injection of VP (1 μg) were not significantly different from those of Long–Evans rats (Burnard et al., 1985). The disturbances consisted of periods of staring and immobility, locomotion difficulties and myoclonic jerks often followed by scratching. After a second i.c.v. injection with 10-ng VP, Di rats exhibited more pronounced motor disturbances. This was not caused by a general decrease in seizure threshold since susceptibility to pentylenetetrazol was similar in HoDi and control rats. As VP facilitates pilocarpine-induced seizures in normal Wistar rats (Croiset and De Wied, 1997), one would have expected a decreased sensitivity of the Brattleboro rat.

Male HoDi rats are unable to maintain copulatory behavior after castration. Intromission and ejaculatory pattern disappear almost immediately after castration. HeDi rats show only a gradual decline of the behavior. If the copulatory behavior of the HoDi rats is normalized by testosterone treatment, and DG-LVP is given immediately after the copulatory session the ejaculatory behavioral pattern is maintained after cessation of the testosterone treatment. These findings were interpreted in terms of the role of VP in the maintenance of a genetically determined social behavioral repertoire that is dependent on experience (Bohus and Koolhaas, 1985).

Electrophysiological studies

Knowledge on the influence of neurohypophyseal hormones on the brain can also be derived from electrophysiological studies (for review see De Wied et al., 1988). Changes in hippocampal theta rhythm reflect a functional state of the brain, which is related to attention, learning, and memory processes. The amount of theta activity substantially increases in some cortical and subcortical areas during learning of an appetitive approach situation (Elazar and Adey, 1967). VP significantly increased peak frequency in hippocampal theta activity during paradoxical sleep (PS) or following stimulation of the mesencephalic reticular formation (MRF) (Urban and de Wied, 1978; Bohus et al., 1978). HoDi rats were therefore employed to investigate the absence of VP on PS and hippocampal theta activity. The absence of VP had virtually no effect on the amount of PS generated during 8 h recording sessions. DGAVP (i.c.v.) did not modify the amount of PS. However, a marked deceleration of theta activity was found in HoDi rats. Mean and peak frequencies were substantially lower in HoDi rats as compared to HeDi and HoNo animals. DGAVP temporarily restored the quality and quantity of theta activity following s.c. as well as i.c.v. administration (Urban and De Wied, 1978). These studies show that the influence of VP as with most neuropeptides can be detected in particular in situations in which the demand on the function of a given structure is increased. The quality of theta activity is closely related to the functional state of midbrain limbic structures. The deceleration of theta activity in reticulo-septal-hippocampal pathways may indi-

cate a decrease in vigilance. Conversely, VP and related peptides that increase mean and peak frequencies may increase vigilance and thereby enhance learning and memory processes.

Electrophysiological investigations performed on hypothalamo-neurohypophyseal neurons of Brattleboro rats did not reveal alterations in firing characteristics as compared to those found in controls (Dreifuss et al., 1982). VP accelerated neuronal firing in the CA1 area in hippocampal slices of homozygous Brattleboro rats not different from that found in slices obtained from Long–Evans rats. These authors concluded that the hippocampal neurons had a normal sensitivity to VP. Accordingly, the genetic defect in VP synthesis does not affect the peptide-dependent excitability and signal transduction on the neurons.

Brief high frequency stimulation of excitatory synapses can induce a long-lasting increase in transmission in these synapses (Bliss and Lomo, 1973). This activity-dependent increase in efficacy of synaptic transmission is known as long-term potentiation (LTP). It can develop in many brain structures including the hippocampus, lateral septum and neocortex (Maren and Baudry, 1995). Most authors agree that LTP and memory processes in the brain are closely connected (Malenka and Nicoll, 1997). The excitatory amino acid glutamate is the putative neurotransmitter involved (Teyler and DiScenna, 1987). Because of its long duration LTP is believed to be the basis of memory processes in the brain. Evidence has been presented that VP and related peptides can induce LTP-like effects in hippocampal slices (Chepkova et al., 1995; Brinton et al., this volume). Field potentials recorded in the lateral septum in Wistar rats before and after acquisition and extinction of shuttle-box avoidance behavior, showed a 20–30% increase in the N-wave after the training in good learners, while the bad learners showed a 10–20% decrease in the N-wave of the field potentials. Rats of the Brattleboro strain with predominantly bad learners showed only a decrease in N-wave after acquisition training presumably caused by the absence of VP. In another study, Urban et al. (1989) showed that high frequency stimulation of the fimbria fibers induces LTP in excitatory transmission between these fimbria fibers and neurons of the lateral septum. Septal slices prepared from HoDi rats brain consistently failed to maintain LTP. In vitro VP superfusion or s.c. administration during several days before the experiment corrected this failure (Van den Hooff et al., 1989). The same phenomenon was observed when LTP was induced in lateral septum slices of Wistar or Long–Evans rats when superfused with d(CH_2)5-Tyr(Me)-Arg-VP a V1 receptor antagonist. These results suggest the importance of VP in maintaining LTP and its role in memory processes. Results however were less clear when LTP was induced in the ventral hippocampus, the most sensitive site of the memory effect of VP, following tetanic stimulation of the Schaffer collaterals in HoDi rats as compared to Long–Evans animals (Van den Hooff, 1989).

VP lowers the seizure threshold in picrotoxin treated Wistar rats (Croiset and De Wied, 1997). Indeed the lack of VP as in the Brattleboro rat or after passive immunization with VP antiserum induced convulsions at higher body temperatures than in untreated Long–Evans animals (Kasting et al., 1981), thus suggesting that VP mediates febrile convulsions. The development of amygdaloid kindling in the Brattleboro rats are also retarded (Gillis and Cain, 1983). The kindling model of epilepsy is also related to plastic synaptic events (Cain, 1989).

Thermoregulation

VP is involved in thermoregulation (for review see De Wied et al., 1993). Potent stimuli, which release VP in sheep and rat and microinjection of VP into the ventral septal area in the rat, attenuate fever induced by bacterial endotoxin. Microinjection of bacterial endotoxin into a lateral ventricle produces fever in Long–Evans but not in HoDi Brattleboro rats. Both strains however produced fever after i.c.v. PGE2 (Eagan et al., 1982). This suggests the involvement of VP in the early phase of the central prostaglandin cascade. The i.v. injection of an endogenous pyrogen induced fever in Brattleboro rats comparable with that found in Sprague–Dawley animals. In contrast, the i.v. injection of a lipopolysaccharide endotoxin, which causes a hypothermic response in normal rats, induced fever in HoDi Brattleboro animals (Stitt and Shimada, 1987). In newborn mammals,

hypoxic-hypoxia produces a decrease in body temperature associated with increased levels of VP in CSF and plasma. This hypothermic response is even accentuated in VP-deficient Brattleboro rats (Clark and Fewell, 1994). Accordingly, it is not clear yet how and where VP is involved in thermoregulation.

Circadian rhythms

The suprachiasmatic nucleus (SCN) of the hypothalamus is the biological clock which determines the circadian rhythms in mammals (Buijs, 1996). The firing rate of SCN neurons peaks during the light phase. Neurons of the SCN represent one of the cerebral vasopressinergic systems. VP is synthesized and released within the SCN with circadian rhythm. VP contributes to the endogenous excitation of SCN neurons. In vitro extracellular recordings in tissue slices of the suprachiasmatic nucleus from homozygous and heterozygous Brattleboro rats displayed a circadian rhythm of spontaneous (basal) activity with firing rates declining during the dark phase. This indicates that the endogenous pacemaker driving the circadian rhythm is not dependent upon the presence of VP. However, the peak of activity displayed during the light phase is significantly lower in the VP-deficient animals (Ingram et al., 1996). The authors suggest that VP functions to amplify the rhythm by its excitatory effect during the light phase. They also showed that a high proportion of neurons in the slices was excited by application of VP indicating that receptors in those neurons were functional. These findings corroborate the conclusions obtained from in vivo electrophysiological studies (Dreifuss et al., 1982).

When Brattleboro rats and Long–Evans animals were exposed to a 1 h presentation of food, both strains of animals were found to exhibit a phase shift of activity and a body temperature rise correlating with the presentation of food. The DI rats developed a diurnal shift more rapidly than the Long–Evans animals (Murphy et al., 1993). This suggests that the rhythmicity in the VP-deficient rat be less fixed than in normal animals. The Brattleboro rats showed a dramatic increase in activity during the light phase. This might be caused by their drinking behavior. In the dark phase, body temperature of the DI rats was lower than that of the Long–Evans rats, whereas in the light phase, it was higher. Apparently the absence of VP interfered with the modulation of thermoregulation. The control rats showed a significant attenuation of activity but maintained both nocturnal and diurnal temperature peaks throughout the food-restricted condition. In a subsequent study, Murphy et al. (1996) found that Brattleboro rats as compared to Long–Evans animals exposed to a 12:12 h light/dark cycle showed nocturnal activity and temperature rhythms during the habituation period when the animals had free access to food and water. The DI rats were more active during the light phase and had a lower body temperature during the dark phase. During the period when the animals were given two 1-h feeding periods, marked changes were observed. In the DI rat the photic oscillator was lost and core temperature, heart rate, and activity shifted from nocturnal to diurnal patterns. Circadian rhythms of the DI rat were now synchronized to the non-photic Zeitgeber of scheduled food presentation. Control rats lost a well-defined rhythmicity from the adherence to the photic oscillator while at the same time adhering to the non-photic Zeitgeber.

Behavioral stress and the hypothalamic-pituitary adrenal axis

Several reports in the literature indicate that the adrenocortical response to stress is impaired in HoDi rats (McCann et al., 1966; Arimura et al., 1967). Smaller adrenal weight point to a deficient adrenal function while the response to stress appeared to be normal or decreased. Plasma corticosterone was found to be somewhat decreased (Sokol and Zimmerman, 1982). Base-line pituitary-adrenal activity of HoDi rats did not differ from that of HeDi and Wistar rats (Bohus et al., 1975). Adrenal glands and testes weights tended to be smaller in both HoDi and HeDi rats as compared to Wistar animals and HoDi rats had a heavier posterior pituitary. A remarkable difference in pituitary-adrenal activity was found in animals during the retention tests of the passive avoidance behavior. Plasma corticosterone measured immedi-

ately after the learning trial was similar in both HoDi and HeDi rats. However, when the rats were tested for retention 3 or 24 h after the learning trial plasma corticosterone of HoDi rats in which avoidance behavior was markedly reduced, responded much less than that of HeDi rats. Thus, retention impairment was accompanied by a diminished pituitary-adrenal response. Treatment with DGAVP restored passive avoidance behavior of HoDi rats, but did not normalize the plasma corticosterone response. Accordingly, the diminished pituitary-adrenal responsiveness of the HoDi rats cannot be simply ascribed to the behavioral deficit. Williams et al. (1983a) showed that the rise in corticosterone in response to exposure to an open field was similar in HoDi and Long–Evans rats, but an acoustic stress resulted in a lower corticosterone response than in controls. They also found that the concentration of norepinephrine, dopamine, and serotonin in limbic regions of HoDi rats was increased. These findings were interpreted as indicating a lower emotionality of the HoDi rat. This according to these authors may explain the memory deficit of the VP-deficient Brattleboro rat.

As VP potentiates CRH activity (Buckingham, 1982) one would expect abnormalities in pituitary-adrenal activity in particular in response to all kinds of stress in animals deficient in VP. However, it does not dramatically affect the corticotrophic function. This as others and we have found depends on the stress used to provoke the response. The adrenal response to a neurogenic stress as measured in terms of plasma ACTH and corticosterone levels was found reduced in HoDi rats as compared to that of Long–Evans animals (Lutz-Bucher and Koch, 1982). Chronic treatment with VP but also with OT restored the response. Murphy and Wideman (1992) found differences in the plasma corticosterone response to food-restriction in the HoDi and normal Long–Evans rats. One-day food-restriction results in marked corticosterone response in both lines with higher levels in Long–Evans rats. The hormonal stress response diminishes in the Long–Evans rats during the subsequent restriction days, whereas the plasma corticosterone levels remain high in the HoDi mutants. The findings of Bohus et al. (1975), that acute treatment with DGAVP which corrected the behavior but not the corticosterone response, indicate that the presence of VP is mandatory for the pituitary-adrenal response to neurogenic stress. It may be that neurogenic stress, e.g. emotion in particular needs the interaction between CRH and VP to evoke the release of ACTH. The results of a replication of these experiments using all three genotypes of the Brattleboro strain is shown in Table 1. Immediately following the inescapable foot shock during learning the rise in plasma corticosterone levels, normally found in Wistar rats, did not occur in HoDi and HeDi rats. However, a significant elevation in wild-type animals was observed. In contrast, plasma corticosterone levels were markedly increased in the HoDi and HeDi rats at the retention test shortly (few minutes) after the learning trial. Surprisingly, a similar elevation did not occur in the wild-type rats despite markedly increased avoidance latencies. Twenty-four hours following the learning trial HoDi rats showed the expected behavioral deficit together with the absence of the pituitary-adrenal response. Both HeDi and wild-type rats displayed avoidance behavior and a significant increase in plasma corticosterone levels. Finally, administration of VP or DGLVP to HoDi rats normalized the retention behavior, but failed to restore the plasma corticosterone response. A partial effect was observed when VP was given shortly before the retention test, but not when DGLVP was administered in the same way, although it corrected the passive avoidance response. The most parsimonious explanation of the findings is again that the presence of VP is essential for the neurogenic release of ACTH. In the mean time, the unexpected observations in the wild-type rats called again the attention to the possibility that some of the impairments in the HoDi rats are not necessarily the consequence of the absence of VP. Therefore, we investigated the plasma corticosterone response of all Brattleboro genotypes to repeated open-field tests and following exposure to ether vapor. The response to open-field behavior was impaired as in the passive avoidance paradigm while the response to ether vapor was more or less the same in the three genotypes (data not shown). These findings reinforce the notion that VP is involved in the pituitary-adrenal response to neurogenic stress.

Table 1
Plasma corticosterone levels in Brattleboro rats in a passive avoidance situation: effect of vasopressin replacement of the homozygous diabetes insipidus rats[a]

Groups	Retention (h)	Plasma corticosterone levels[b]		
		HoDi	He	Wild
Control	–	8.3 ± 0.7	10.5 ± 1.1	9.5 ± 0.8
Trained	–	7.4 ± 0.4$^{\S, ¶}$	10.5 ± 1.1	13.7 ± 1.0*
Control	0	9.4 ± 2.5 (9.5)	8.6 ± 0.5 (9.5)	11.8 ± 1.0 (8.5)
Trained		26.4 ± 0.6**¶ (287)	25.0 ± 1.3**§§ (300)	14.3 ± 0.7 (300)
Control	24	10.6 ± 1.9 (15)	12.2 ± 2.0 (6)	10.7 ± 0.4 (22)
Trained		12.4 ± 1.6$^{\S ¶ ¶}$ (9.5)	23.5 ± 1.8**§ (300)	16.7 ± 1.9* (202)
VP PL	24			
Control		12.3 ± 1.4 (9)		
Trained		14.0 ± 1.2 (300)		
VP PR	24			
Control		8.3 ± 1.1 (21)		
Trained		13.6 ± 1.2** (300)		
DGLVP PL	24			
Control		11.9 ± 1.5 (13)		
Trained		10.9 ± 1.6 (300)		
DGLVP PR	24			
Control		7.8 ± 1.3 (10)		
Trained		7.4 ± 1.0 (240)		

[a] Trained rats were subjected to pretraining and received a single unavoidable foot shock of 1.0 mA of 3 s duration. Control rats were subjected to pretraining procedure, but did not receive foot shock. Passive avoidance latencies (s) are in parentheses. HoDi, homozygous diabetes insipidus (−/−) rat; He, heterozygous (+/−) rat; Wild, wild-type (+/+) rat; VP, arginine-vasopressin (1 μg/rat); DGLVP, desglycinamide-lysine-vasopressin (1 μg/rat); PL, treatment s.c. immediately after the learning trial; PR, treatment s.c. 60 min before the retention test. Statistics (*t*-test): $*P < 0.05$, $**P < 0.01$ (vs. control; *t*-test). $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ (vs. wild-type; *t*-test). $^{¶}P < 0.05$, $^{¶¶}P < 0.01$ (vs. He; *t*-test).

[b] In μ g/100 ml plasma.

Endocrine abnormalities

Many endocrine abnormalities were found in the HoDi rat. These may be secondary effects of the deficiency in VP, but primary genetic disturbances cannot be excluded. Replacement therapy with exogenous VP corrects the high fluid turnover in these rats as well as some but not every endocrine abnormality found in these rats.

Sokol and Zimmerman (1982) summarized the various endocrine abnormalities found in the Brattleboro rat. The levels of OT and its neurophysin, TSH, FSH, renin, AngII in plasma were found increased and the levels of ACTH, aldosterone, corticosterone and the thyroid hormones decreased. The levels of GH, PRL were similar to those found in control Long–Evans rats. Tissue levels of OT and its neurophysin (in posterior pituitary) GH and PRL (in anterior pituitary) were lower, while levels of TSH and LH were found similar and FSH increased. Pituitary ACTH level were either similar or lower. The same was found for CRF activity in the median eminence, while the levels of GHRF and LRH were of the same magnitude as those of control animals. Results however varied from report to report. Several authors, among others we have found retarded growth by us (Bohus et al., 1975). This may be explained by lower concentrations of GH in the adenohypophysis. Although Fujimoto and Hedge (1982a) found lower plasma PRL

levels, Adler and Sokol (1982) reported that basal circulating levels of GH and PRL of Brattleboro rats did not differ from those found in Long–Evans animals. Also pituitary concentration of these hormones was not different. Thyroid abnormalities as indicated by lower circulating levels of thyroid hormones have been detected in Di rats, which is associated with a compensatory increase in the level of TSH in plasma. This could not be corrected by treatment with VP or DDAVP (Fujimoto and Hedge, 1982b). These authors also found lower PRL levels in plasma. Serum and pituitary concentration of FSH was found decreased in the Brattleboro rat as compared to Long–Evans animals but LH levels in serum and pituitaries did not differ (Opescu et al., 1982). Boer et al. (1982) reported on the fertility and development of Brattleboro rats. Gestation length was shorter, labor more rapid and litters were smaller. The growth of neonates is stunted. Brain growth is impaired in particular the cerebellum but also the medulla oblongata and the cortex. These developmental disturbances could not be corrected by chronic postnatal treatment with long-acting VP preparations (Boer et al., 1984).

Discussion

The Brattleboro rat can be considered a natural knockout animal, which was the first one, which became available for investigation. The genetic disturbance in the generation of VP caused a central diabetes insipidus comparable to that found in humans. The animal appeared to be useful to determine the peripheral and central consequences of VP deficiency. A wealth of information has been collected to demonstrate the peripheral role of VP in diverse physiological functions (Sokol and Valtin, 1982). The present review shows that the genetic defect in the availability of peripheral, but more importantly central VP causes a large number of deficits in the nervous functions. The extensive studies of the behavior of the Brattleboro rats as summarized in Table 2 show that a substantial number of behavioral processes from cognition to drug tolerance are effected in the absence of VP. Behaviorally speaking, it is unlikely that relatively simple explanations as changes in arousal, activation or attention can explain all behavioral deficits of the Di rat. Learning and memory processes in Di rats are mostly impaired in paradigms in which emotional processes are involved, whereas spatial learning and memory is not as seriously damaged. Some behaviors remain unchanged or the dependence of the deficit from the absence of VP is not clearly demonstrated. As recently pointed out by Crawley and Paylor (1997), the absence of changes in behavior may be explained by the unimportance of a (VP) gene for a given behavioral process or that compensation occurs by the expression of other genes. Compensation may occur since both the magno- and parvocellular vasopressinergic cells in the brain co-express a large number of other neuropeptides that are behaviorally active (Kovács and de Wied, 1994). One should however consider that the absence of VP seems to impair the expression of these neuropeptides (Van Leeuwen, 1992). As described earlier in this review prolactin and opiomelanocortin fragments related to enkephalin or ACTH may correct for the behavioral deficit of the Di rat. These peptides may be of pituitary origin as well.

It is more difficult to explain inconsistencies in the findings regarding learning and memory in aversive situations. Differences between breeding lines are not unknown to behavioral geneticist (e.g. Blizard, 1981). Unfortunately, a direct comparison of all breeding lines at least in one behavioral paradigm is not available. Another factor may be sex and age. That the forebrain vasopressinergic system is sexually dimorphic and dimorphism exists in a number of behaviors underline the importance of sex. Furthermore, an age dependent 'spontaneous' shunt occurs in certain cells of hypothalamic magnocellular system of Di rats to a +/− phenotype. However, the shunt is not sufficient to compensate for the diabetes insipidus of the rats, and a similar shunt in the parvocellular extrahypothalamic cells has not been yet demonstrated. The use of a restricted number of genotypes in studies with knockout animals should be seriously considered. To investigate the behavioral phenotypes of knockout (−/−) mice the use of the wild-type (+/+) and heterozygous (−/+) littermates from the F1 heterozygote matings seems to be mandatory (Crawley and Paylor, 1997). If one

Table 2
Behavioral changes in the Brattleboro rats homozygous for hereditary diabetes insipidus: restoration of the alterations by vasopressin or related peptides[a]

Learning and memory	Brattleboro rats	Effect of vasopressin	Reference
Avoidance acquisition: spaced trial	0/−	ND/+	Bohus et. al. (1975, 1978, 1993); Gaffori et al. (1985); Celestian et al. (1975)
Massed trials	−	ND	Drago and Bohus (1986)
Single trial	0	ND	Bohus et al. (1975, 1978, 1993)
Avoidance extinction: spaced trials	−	ND/+	Bohus et al. (1975, 1978, 1993); Gaffori et al. (1985)
Avoidance retention: single trial	−/0	+	De Wied et al. (1988); Bailey and Weiss (1979); Van Haaren et al. (1985)
Approach-avoidance conflicts	+	ND	Brito et al. (1981, 1982); Herman et al. (1986a,b)
Discrimination learning	−	ND	Brito et al. (1981, 1982)
T-maze avoidance learning	0	ND	Brito et al. (1981, 1982); Gaffori et al. (1985)
Reference memory	−	+	Brito et al. (1981, 1982); Gaffori et al. (1985)
Open field long term habituation	−	ND	Bohus et al. (1975, 1978, 1993); Gaffori et al. (1985)
Delayed alternation	−	ND	Colombo et al. (1992)
Conditioned freezing behavior	−	ND	Stoehr et al. (1993)
Social recognition memory	−	+	Engelmann and Landgraf (1994)
Rewarded operant response	−	ND	Brito (1983, 1985)
Other behaviors			
Locomotor activity	+	−	Bohus et al. (1975, 1978, 1993); Williams et al. (1983a,b)
Goal approach behavior	−	ND	Brito et al. (1981, 1982)
Analgesia	−	+	Bodnar et al. (1980, 1982)
Response to electric shock	+	ND	Bohus et al. (1975, 1978, 1993); Bodnar et al. (1980, 1982)
Startle response	+	0	Gash et al. (1982); Warren and Gash (1983)
Development of tolerance: opiates	−	+	De Wied and Gispen (1976)
Alcohol	−	+	Pittman et al. (1982); Hoffman et al. (1979)
Behavioral sensitization: cocaine	−	+	Post et al. (1982)
Motor disturbance: myoclonic jerks	+	−	Burnard et al. (1985)
Circadian activity: pacemaker	0	0	Murphy et al. (1993, 1996)
Photic oscillator	−	+	Murphy et al. (1993, 1996)
Male postcastration behavior	−	+	Bohus and Koolhaas (1985)

[a] −, inhibited, attenuated; 0, no change; +, increased, facilitated; ND, not determined.

scrutinizes the different studies there remain very few of them fulfilling this criterion. Many experiments employed Long–Evans rats from the original parent strain of the Brattleboros as the only control. The breeding history of the recent strain is however entirely different. The use of the albino Wistars cannot be taken anymore as a proper control of the pigmented Brattleboros. Rather, we may consider their data as internal control of the behavioral tests. Additionally, methodological description by the authors does generally not allow precise identification of the genotype of the controls.

Finally, one may not underestimate the role of methodological factors as the source of discrepancies. The differences in training, and notions of the various authors and their approach to the study of

behavior may well be another reason for these discrepancies. This is suggested from results on VP and related neuropeptides on learning and memory processes in intact rats, which suffered from the same controversies. To study the behavior of rodents one needs to know the history of the animals which one is going to use, the way they were bred, housed and handled before used in the experiments. In fact we had the most consistent results with our Wistar strain when we bred our own animals. In addition, differences in behavior are subtle and often found only under mild stimulus conditions. For example, hypophysectomized rats are deficient in acquiring shuttle-box avoidance behavior (De Wied, 1969). However, if shock intensities during training were increased, the behavior looked normal (Fekete et al., 1983). In general, we have used low shock intensities in our conditioning experiments because we experienced that high shock intensities often induce freezing which impairs acquisition of avoidance behavior.

Interestingly, the attenuation in the development of tolerance to opiates and ethanol in HoDi rats which could be restored by treatment with VP or related peptides shows that the VP knockout rat is a useful model to explore the central functions of VP. Results on tolerance development in normal rats however were much less controversial probably because tolerance is an easier measure of behavioral plasticity than avoidance behavior (for review see Kovács and De Wied, 1994). The same holds for the analgesic influence of VP and the hyperalgesia found in Brattleboro animals. On the other hand, the absence of a temperature response to a bacterial endotoxin which needs VP is puzzling since VP in Wistar animals counteracts fever induced by such agents (Kovács et al., 1992). This finding is not in agreement with the effects of VP and related neuropeptides on temperature regulation (Kasting et al., 1980).

In summary, the Brattleboro rat has been a useful model to study the consequences of VP deficiency on water metabolism and the control of the disease e.g. the peripheral endocrine role of the VP hormone. It has also been fruitful in exploring its implication in pituitary function in particular pituitary ACTH release. More importantly, it helped to explore the CNS effects of VP. The abnormalities found may not all be ascribed to VP deficiency per se but to additional hormonal defects which are a consequence of the abnormal VP precursor processed in the peptidergic neurons. The abnormal VP processing also interferes with the expression of a number of co-localized neuropeptides. A knockout model which exclusively lacks the synthesis and release of VP solely in the parvocellular neurons originating in extrahypothalamic sites would be the most adequate model to explore the CNS effects of VP and related peptides. These extrahypothalamic sites such as the BNST, MeA, and their projection areas like the lateral septum, central amygdala, hippocampus, etc. are the loci of most of the central effects of VP (De Wied et al., 1993). Another possibility would be to restore local VP synthesis in the neurons by injection of an appropriate vector. The success of such gene therapy in the −/− Brattleboro rat has been reported recently. Injection of an adenovirus expressing VP into the SON reversed hypothalamic diabetes insipidus in the Brattleboro rats (Geddes et al., 1997). A few words of caution may be in place here. If one would succeed in making a knockout animal with only one deficiency in the synthesis of a particular neuropeptide, one should not be as naive as we were to expect a clear deficiency. Multiple neuropeptides affect the various CNS functions as learning and memory, maternal, sexual, social and other behaviors, or a particular endocrine (pituitary) or metabolic (food and water intake) function. However, if the role of a neuropeptide on CNS function has been thoroughly studied in intact rats, like with VP, one might learn much from such studies.

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SECTION 6

Human brain vasopressin systems

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
Progress in Brain Research, Vol. 119

CHAPTER 6.1

The human hypothalamo-neurohypophysial system in health and disease

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The present paper reviews the changes observed in the human supraoptic (SON) and paraventricular (PVN) nuclei, and their projections to the neurohypophysis, median eminence and to other brain areas in health and disease.

The supraoptic (SON) and paraventricular (PVN) nuclei (SON, PVN)

One of my anatomist friends who has made notable contributions to our knowledge of the anterior lobe hormones has challenged me to produce any corresponding clinical or experimental evidence of posterior lobe activity. To this challenge, this and the succeeding papers are a partial answer.

H. Cushing, 1932, p. 60

The hypothalamo-neurohypophysial system

The supraoptic (SON) and paraventricular (PVN) nuclei (Fig. 1) and their axons running to the neurohypophysis form the hypothalamo-neurohypophysial system (HNS) that represents the classical example of a neuroendocrine system. The SON and PVN are supplied with unusually rich capillary beds. The density of this capillary bed would decrease with age in the PVN, but not in the SON (Abernethy et al., 1993). The decrease in the PVN is, however, rather unexpected and needs confirmation, as a stability was found in the number of oxytocin neurons in aging (Wierda et al., 1991) and even an activation of corticotrophin-releasing hormone (CRH) and vasopressin neurons (Raadsheer et al., 1994a,b; Van der Woude et al., 1995). In order to establish the proportion of SON and PVN cells that project to the neurohypophysis, Morton (1961) determined neuronal numbers in these nuclei for a period of 12–45 months following hypophysectomy, an operation performed in those days as a palliative measure in the treatment of hormone-dependent metastatic mammary carcinoma (see also below). After hypophysectomy there was an average loss of neurons from both the SON and PVN of over 80%. From this observation it was concluded that most neurons of the SON and PVN project to the neurohypophysis. Since the apparent lack of retrograde changes in the accessory SON cells, the islands between the SON and PVN, it seems likely that their axons project more proximally to the stalk of the pituitary (Morton, 1961). Following hypophysectomy or transsection of the stalk it took until about a year after the operation for the stump of the stalk to be innervated again throughout (Daniel and Prichard, 1972).

The SON is subdivided in three parts. The largest part, the dorsolateral SON (Fig. 1), has a volume of 3 mm^3 (Goudsmit et al., 1990) and contains 53 000 neurons, 90% of which contain vasopressin and 10% oxytocin, mainly in its dorsal part (Fliers et al., 1985; Fig. 2). Indeed, J. Purba (unpublished data) counted 49 240 vasopressin and 5460 oxyto-

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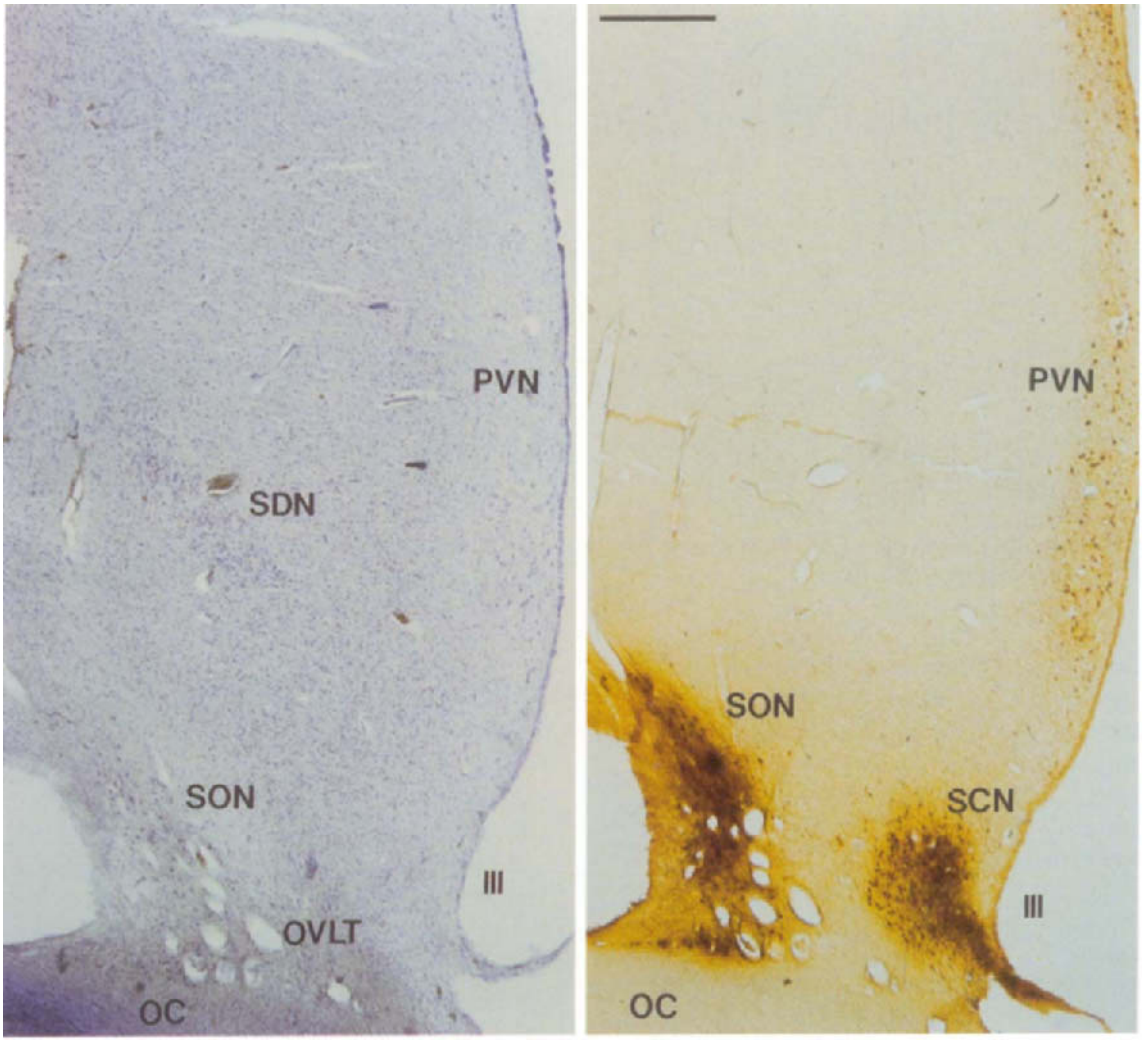


Fig. 1. Thionine (left) and anti-vasopressin (right) stained section through the chiasmatic or preoptic region of the hypothalamus. OC, optic chiasm; OVLT, organum vasculosum lamina terminalis; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SDN, sexually dimorphic nucleus of the preoptic area (intermediate nucleus); SON, dorsolateral part of the supraoptic nucleus; III, third ventricle. Scale bar, 1 mm.

cin neurons in this part of the SON. The dorsomedial and ventromedial SON together contain some 23 000 neurons (Morton, 1961). According to Dierickx and Vandesande (1977) 85% of the neurons of the medial part of the SON contain vasopressin and 15% oxytocin. The entire SON thus contains some 76 000 neurons on one side (Morton, 1961). The PVN has a volume of 6 mm^3 (Goudsmit et al., 1990) and was estimated to consist of about 56 000 neurons (Morton, 1961) of which some 25 000 contain oxytocin and 21 000 express vasopressin (Wierda et al., 1991; Purba et al., 1993; Van der Woude et al., 1995). The estimate of the exact neuron numbers in the SON and PVN depends, however, strongly on the methods used (Harding et al., 1995).

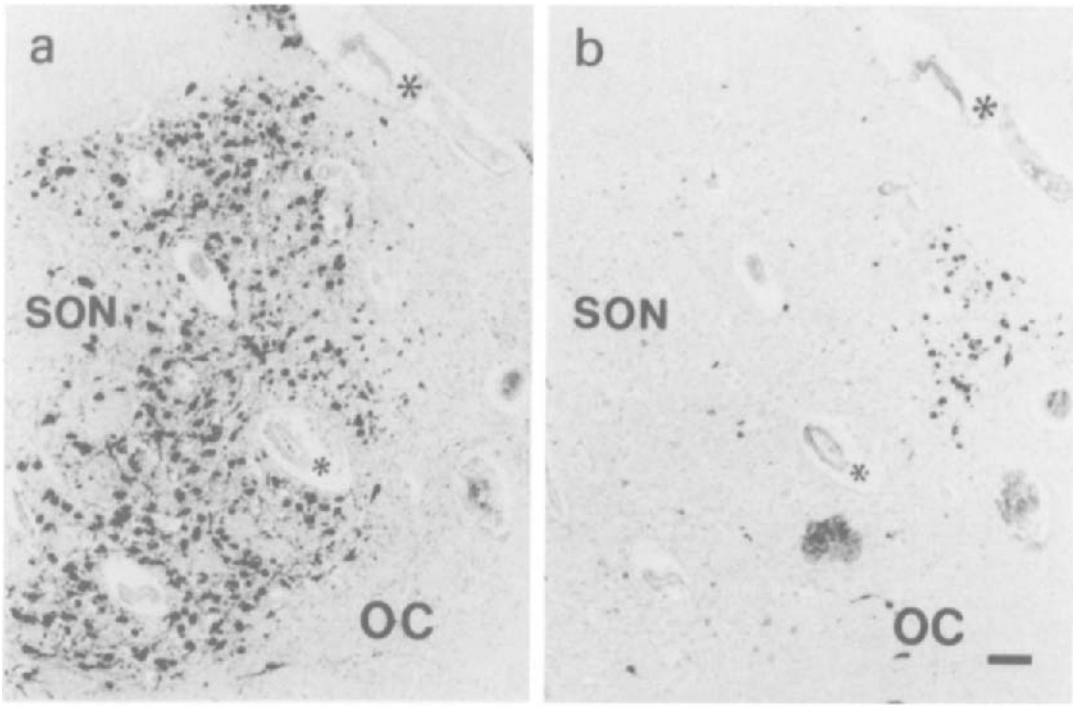


Fig. 2. Consecutive sections of a 49-year-old female control stained for vasopressin and oxytocin. (A) Dorsolateral supraoptic nucleus (SON) stained with an antiglycopeptide (Boris Y-2) against the vasopressin precursor and (B) oxytocin staining (0-1-V, purified). Note that the relatively small oxytocin cell population is clearly separated from the vasopressin cell population. Asterisk indicates a blood vessel that is present in both consecutive sections: OC, optic chiasm. Scale bar, 100 μm (from Evans et al., 1996, with permission).

Neurosecretion

In the 1940s practically everybody vigorously, or even viciously, rejected the concept of neurosecretion.
(Bertha Scharrer, personal communication).

For a long time it was believed that the neurohypophysis made a product and that this function was regulated by the innervating nerves. In 1908, P.T. Herring first described the 'peculiar hyaline bodies' seen in sections of the neurohypophysis and expressed the belief that they represented the secretory product of the epithelial investment of the posterior lobe known as the pars intermedia. The globules appeared to find their way toward the tuber cinereum and 'in favorable histological preparations could be seen passing between the bodies of the ependymal cells to enter the infundibular cavity' (Cushing, 1932; p. 59). A famous case report tells of a man who, in 1910, received a gunshot wound, resulting in a polyuria of 97 liters a day and sexual dystrophy. At autopsy the bullet was found to be lodged in the sella-turcica and to have destroyed the infundibular process and posterior lobe (Vonderahe, 1940; Brooks, 1988). Although such observations revealed the antidiuretic function of the neurohypophysis, the concept of 'neurosecretion' in vertebrates as found in the large neurons of the human supraoptic and paraventricular nucleus, was not proposed until 1939, by the Scharrers (Scharrer and Scharrer, 1940; Brooks, 1988; Meites, 1992). According to the critics of those days, this concept was based on 'nothing more than signs of pathological processes, postmortem changes or fixation artifacts'. In the 1940s 'practically everybody vigorously or even viciously' rejected the concept that a neuron

could have a glandular function (B. Scharrer). This initially highly charged reception of the neurosecretion concept was followed by acceptance only when Bargmann (1949) demonstrated the same Gomori-positive material in the neurohypophysis and in the neurons of the SON and PVN and concluded that the axons from the SON and PVN transport material to the neurohypophysis. He called the aggregate of fibers 'the neurosecretory pathway'.

> *Seeking a new method for revealing neurosecretory material, he (Bargmann) placed sections from a dog's brain into acid-permanganate-chrome-alum-hematoxylin, according to Gomori's method... and was astonished at what he found (instead of shouting Eureka, Bargmann, waving the cigar that was always in his hand exclaimed (magna voice): 'Donnerwetter!'), the cells of the supraoptic and paraventricular nuclei and the fibers extending into the infundibulum and reaching the posterior lobe had selectively taken on a blue hue! ... When, everafter, in papers dealing with hypothalamic neurosecretion 'Gomori-positive' and 'Gomori-negative' results were cited, Gomori would comment in conversation that he found the terms distasteful but amusing. 'Right now' he once said before lunch to a fellow-Hungarian, Jacob Furth: 'I feel Gomori-negative'*
>
> *(Anderson and Haymaker, 1974).*

In 1955, Dr. Vigeaud received the Nobel Prize for the elucidation of vasopressin and oxytocin and the synthesis of vasopressin. The nonapeptides vasopressin and oxytocin are synthesized as part of a large precursor that includes a neurophysin for both peptides and a c-terminal glycoprotein for vasopressin. The vasopressin and oxytocin precursor genes are only separated by 12 Kb in the human genome. The genes are located on the distal short arm of chromosome 20 (Schmale et al., 1993). When the neurophysins were discovered by Asher et al. they were recognized as the inactive fragments of the precursor with a higher molecular weight and were thought to act as carriers for vasopressin and oxytocin. The role of neurophysins is at present also considered in the light of the knowledge on mutations in the neurophysin part, causing diabetes insipidus. Any disruption of the structure and thus of the three-dimensional conformation of neurophysins by mutations may cause a decline in the binding and activity of endopeptidases responsible for the cleavage of vasopressin. Mutations in the neurophysins may also produce a change in the polymerization of neurophysins and salt bridges, with the result that there may be, e.g. an accelerated aspecific enzymatic degradation of the hormone revealing the clinical symptomatology. So rather than being a mere inactive part of the precursor, neurophysins are now considered as an essential system for carrying and protecting the non-apeptides (Legros and Geenen, 1996). This concept is supported by observations on a Dutch family with hereditary hypothalamic diabetes insipidus, based upon a single G to T transversion in the neurophysin-encoding exon B (Bahnsen et al., 1992). When this mutant DNA was stably expressed in a mouse pituitary cell line, the mutant precursor was synthetized but processing and secretion were dramatically reduced and the protein did not seem to reach the trans-Golgi network (Olias et al., 1996). Other studies on reversal mutant AVP genes showed accumulation of mutant AVP precursors in the endoplasmic reticulum and reduced viability of the cell lines (Ito et al., 1997; see below).

Vasopressin and oxytocin: Production and release

Vasopressin is synthesized in the SON and PVN (Dierickx and Vandesande, 1979), suprachiasmatic nucleus (SCN; Swaab et al., 1985), diagonal band of Broca (DBB), nucleus basalis of Meynert (NBM) (Ulfig et al., 1990), and bed nucleus of the stria terminalis (BST) (Fliers et al., 1986; Mai et al., 1993), and oxytocin in the PVN and the dlSON (Dierickx and Vandesande, 1979) (Figs. 1–3). Vasopressin and oxytocin are produced in different neurons (Dierickx and Vandesande, 1979; Hoogendijk et al., 1985). The observation in rat that under extreme forms of stimulation the neurons may produce both peptides (Mezey and Kiss, 1991) has not been followed up in humans yet. A rostrocaudal gradient in the ratio between vasopressin and oxytocin neurons is present in the PVN. Whereas the ratio of vasopressin: oxytocin cells remained 80% from rostral to caudal over a

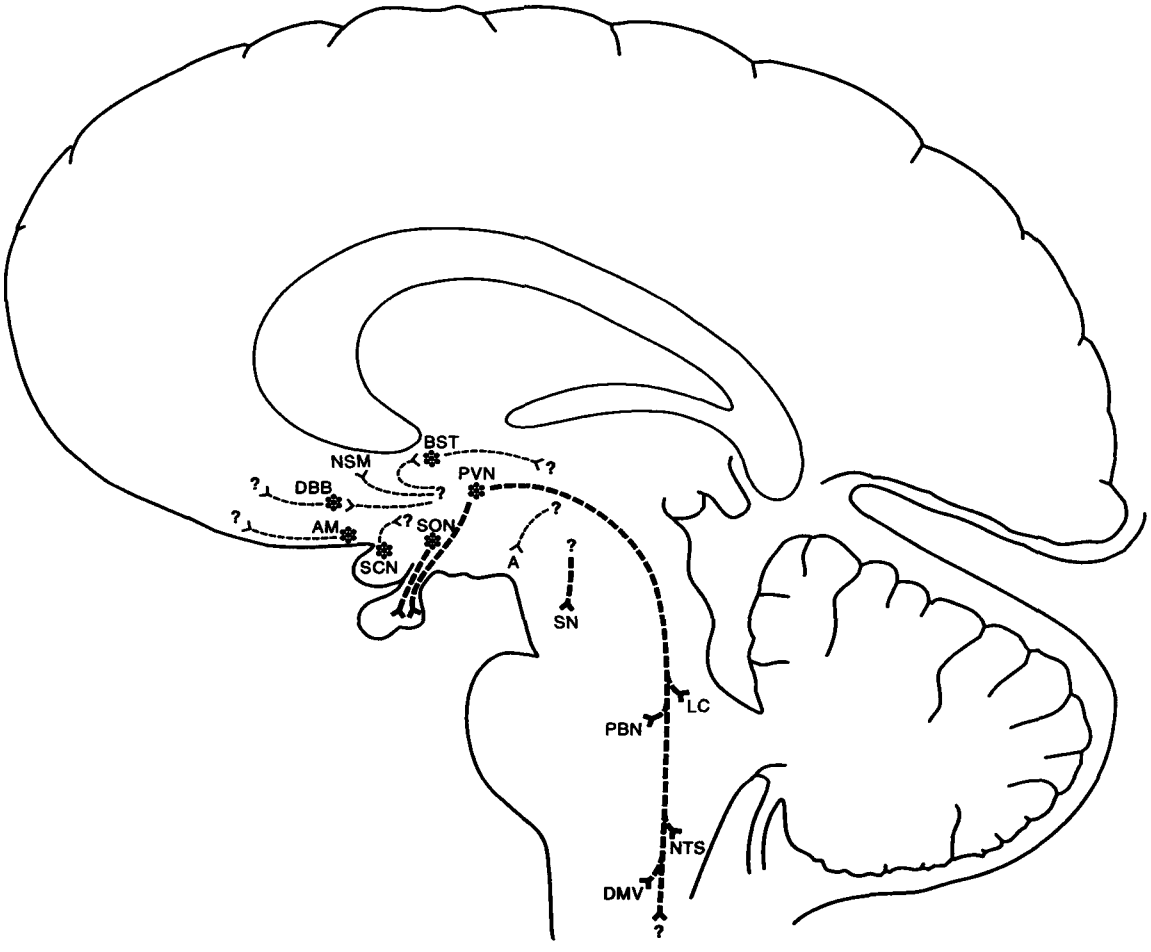


Fig. 3. Vasopressin pathways in the human brain. Question marks indicate that at present no site of origin or termination is known. (A) Amygdala: AM, anteromedial subnucleus of the basal nucleus; BST, bed nucleus of the stria terminalis; DBB, diagonal band of Broca; DMV, dorsal motor nucleus of the nervus vagus; LC, locus coeruleus; NSM, nucleus septalis medialis; NTS, nucleus of the solitary tract; PBN, parabrachial nucleus; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SN, substantia nigra; SON, supraoptic nucleus (scheme from E.J. van Zwieten).

distance of 1.5 mm in the dlSON. In the PVN this ratio starts below 20% rostrally, goes up to 60% in the caudal half, after which the ratio decreases again (Swaab et al., 1987b). Recently Mai et al. (1993) pointed to an additional oxytocin containing cell group dorsolateral to the fornix which they refer to as the parafornical cell group. It is not clear why this cell group should not be considered as part of the PVN. Clusters of magnocellular neurosecretory neurons containing oxytocin or vasopressin are also found in the hypothalamic gray in between these nuclei. These ectopic clusters, which tend to be arranged around blood vessels, are generally referred to as 'accessory nuclei'. They contain more oxytocinergic neurons than vasopressinergic ones (Dierickx and Vandesande, 1977). However, in the older literature, Feremutsch (1948) called the scattered cells and islands of neurosecretory cells between the SON and PVN 'intermediate nucleus'. This is a confusing term, since Brockhaus (1942) originally used the same name for the sexually dimorphic nucleus

of the preoptic area (SDN-POA) (Braak and Braak, 1992). Because male subjects have higher vasopressin plasma levels than females (Van London et al., 1997; Asplund and Åberg, 1991), sex differences in the hypothalamo-neurohypophysial system were expected to be present. And indeed we recently found an increased activity of vasopressin neurons in the dlSON of young males as compared to young females (unpublished data).

Neuroendocrine functions and factors affecting vasopressin and oxytocin release

Vasopressin and oxytocin released into the blood circulation act as neurohormones. In humans, 90% of the circulating vasopressin bound to platelets (Preibisz et al., 1983; Bichet et al., 1986; Nussey et al., 1986; Van der Post et al., 1994) and a patient with autoimmune thrombocytopenia (D.F.S., unpublished data) had indeed low total vasopressin levels in the platelet fraction. Vasopressin acts as an anti-diuretic hormone on the kidney and regulates freewater clearance by V2-type vasopressin receptors coupled to aquaporins (Knepper, 1994). In spite of the suggestions of Cushing (1932) that there would be no clinical or experimental evidence in those days of posterior lobe function (see citation at the beginning of this chapter), endocrine effects on the kidney and uterus had already been known for years. In 1913 Von den Velden and Farini already described the antidiuretic effect of posterior pituitary extracts in patients suffering from diabetes insipidus and in 1909 Blair-Bell reported the oxytocin effects of posterior pituitary extracts in labor.

The hypothalamus integrates signals from osmoreceptors that are probably localized in the organum vasculosum lamina terminalis and stretch receptors of the vascular tree. Predominant stimuli for thirst and vasopressin release in human are osmolality of the extracellular fluid and hypovolemia. Suppression of thirst sensation and decreased vasopressin secretion are probably mediated by stimulation of oropharyngeal receptors and/or distension of the stomach. In this way, plasma osmolality is regulated within narrow boundaries of 2–3 mosmol/kg (Jenkins, 1991). Osmotic stimuli cause a release of vasopressin (Kirkland et al., 1984; Helderman et al., 1978; Phillips et al., 1984; Robertson and Rowe, 1980). Moreover, orthostasis, smoking and nicotine cause vasopressin release (Rowe et al., 1980) and alcohol inhibits vasopressin release in young subjects (Helderman et al., 1978). Chronic alcohol consumption causes a loss of vasopressin neurons (Harding et al., 1996). Morphine gives a release of vasopressin, whereas naloxon is without effect on basal vasopressin levels in man (Van Wimersma Greidanus and Grossman, 1991). In urine, vasopressin shows a nocturnal increase in levels. In enuretics, however, this normal diurnal rhythm is absent (Rittig et al., 1989; see below). Plasma vasopressin is reported to be lower during menstruation than at ovulation (Forsling et al., 1981). In addition, vasopressin release is inhibited by glucocorticoids and stimulated in case of adrenal insufficiency (Ahmed et al., 1967; Erkut et al., 1998).

In women, oxytocin is involved in labor and lactation. During normal delivery, stretching of the lower birth canal is thought to trigger the neurohormonal 'Ferguson' reflex, leading to rapid secretion of oxytocin by the pituitary gland, which results in strong expulsive contractions. The Ferguson reflex is blocked by epidural analgesia (Goodfellow et al., 1983), while alcohol, too, inhibits oxytocin release during labor (Thornton et al., 1992). A significant increase in oxytocin blood levels is found at the onset of full cervical dilatation and crowning of the fetal head, but this increase does not occur following a lumbar epidural block. This explains why, following epidurals, more forceps deliveries are required. Indeed, oxytocin treatment during the second stage of labor with epidural analgesia reduces the need for forceps (Goodfellow et al., 1983). Since a competitive analogue of oxytocin inhibits contractions in uncomplicated preterm labor (Turnbull, 1987; Åkerlund et al., 1987) oxytocin might be involved in the initiation of preterm labor. It should be noted though that more recent research shows that a marked increase of oxytocin blood levels occurs only in a minority of the patients with a normal delivery. Progress of labor was not found to be related to an increase in oxytocin blood levels in that study either, which does not support a role for oxytocin during spontaneous at-term labor (Thornton et al., 1992). However, changes in oxytocin and

vasopressin (V_1) receptors could also modify uterine activity. Such receptors are present in pregnant human myometrium and decidura (Ivanisevic et al., 1989). Only oxytocin (not vasopressin) receptors are thought to increase during pregnancy in the human uterus (Tence et al., 1990). In fact, uterine oxytocin receptors peak in early labor (Maggi et al., 1990), suggesting a role in parturition. As shown by the presence of oxytocin mRNA in human amnion, chorion, and decidua, oxytocin is also produced in these tissues, while oxytocin gene expression increased 3–4 fold around the time of onset of labor. The possible role of this extrahypothalamic oxytocin in the onset and course of labor that would not be reflected in increased blood levels is, however, not yet established (Chibbar et al., 1993). The putative role of fetal vasopressin and oxytocin in parturition is discussed below.

Neurotransmitters and neuromodulators

> *... and evidence will be presented to show that posterior-lobe extracts are far more potent when injected into the cerebral ventricles than by any other means of administration.*
>
> *(Harvey Cushing, 1932, p. 21)*

Commercially available posterior lobe extract ('obstetrical pituitrin') which Cushing, 1932, p. 59) injected into the ventricle in 38 instances in 24 subjects convalescing from operations for pituitary adenoma, appeared to have a pronounced stimulatory effect, essentially parasympathetic in character, and, apparently, central in origin (Fig. 4). Since intramuscularly injected pituitrin had an opposite effect, and no central effects were noted when the hypothalamus was affected by hydrocephaly or by a tumor, Cushing indeed had solid evidence to propose a central action (i.e. on the hypothalamus) of neurohypophysial extracts. These impressive observations passed into oblivion, perhaps because the scientific community chose to put every effort into refuting the 'neurosecretion' concept proposed by Scharrer and Scharrer (1940). The initially highly charged reception of the neurosecretion concept was followed by acceptance only when Bargmann (1949) demonstrated the same Gomori-positive material in the neurohypophysis and in the neurons of the SON and PVN. In the concept of neurosecretion, the proposition of Barry (1954) concerning the existence of Gomori-positive endings outside the hypothalamus ('synapses neurosécrétoires') could not be properly appreciated and was, therefore, eventually forgotten, which is likely to have been expedited by the fact that his articles were in French.

When De Wied (1965) showed that posterior lobectomy in the rat resulted in an accelerated extinction of conditioned shuttlebox avoidance response, and, later on, that this behavioral deficiency could be alleviated by peripherally adminis-

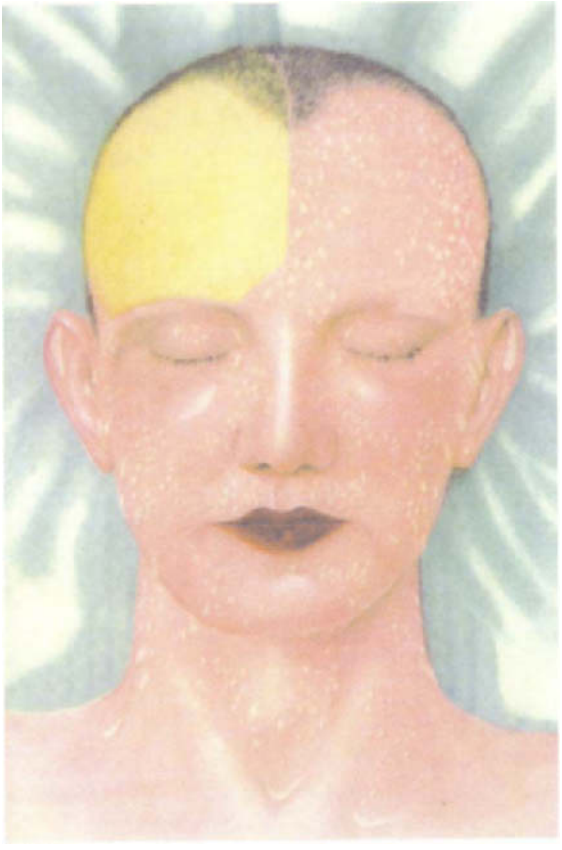


Fig. 4. Showing the vaso-dilatator and suderific effects, sparing the bone flap of the recent operation, of 2.5 mg of pilocarpine injected into the cerebral ventricles: an intraventricular injection of one ml of pituitrin in susceptible persons gives an equally marked response (Cushing, 1932, p. 58).

tered vasopressin (De Wied and Bohus, 1966), it was also only natural that these effects were explained on the basis of the release of neurohypophysial hormones into the bloodstream, which in turn would transport the peptides back to the brain. However, a number of observations have made it extremely unlikely that the vascular route or the cerebrospinal fluid (CSF), would be a major channel for the central actions of neurohypophysial hormones (Swaab, 1982; Swaab et al., 1987a; Kruisbrink et al., 1987). In the meantime, moreover, immunocytochemical observations had revealed yet another site of production for vasopressin, the suprachiasmatic nucleus (SCN) (Swaab et al., 1975). Immunocytochemistry subsequently led to the rediscovery of extensive extrahypothalamic pathways terminating in structures ranging from the olfactory bulb to the spinal cord (Swanson, 1977; Buijs et al., 1978; Sofroniew and Weindl, 1978). The main sources of these vasopressinergic and oxytocinergic pathways were thought to be the SCN and the PVN, although the SON could not be excluded altogether as an additional site of origin. Later, however, colchicine treatment has revealed vasopressin-containing neurons in the rat bed nucleus of the stria terminalis (BST), amygdala, dorsomedial hypothalamic nucleus, locus coeruleus, and septum (Caffé and Van Leeuwen, 1983; Van Leeuwen and Caffé, 1983). Previously, the putative source of the fiber projections had been established only by tracing immunocytochemically stained fibers in serial sections. However, this method proved to be unreliable because, when vasopressin projections from the SCN were checked by lesioning this nucleus, it turned out not to be the source, e.g., of the lateral septum VP innervation, but other proposed target regions of the SCN, viz., the dorsomedial nucleus of the hypothalamus and the organum vasculosum of the lamina terminalis, were confirmed (Hoorneman and Buijs, 1982). Ultrastructural immunocytochemical observations in rat have shown that extrahypothalamic peptidergic fibers terminate synaptically on other neurons (Buijs and Swaab, 1979).

In the human brain vasopressin and oxytocin fibers probably originating in the PVN, project, e.g. to the nucleus basalis of Meynert (NBM), the diagonal band of Broca (DBB), septum, BST, locus coeruleus, the parabrachial nucleus, the nucleus of the solitary tract, the dorsal motor nucleus of the nervus vagus, substantia nigra, dorsal raphe nucleus and spinal cord (e.g. Fliers et al., 1986; Sofroniew, 1980; Unger and Lange, 1991; Fodor et al., 1992; Van Zwieten et al., 1994, 1996; Fig. 3). In addition, vasopressin-containing fibers innervate the fissures of the chorioid plexus in rat (Brownfield and Kozlowski, 1977), while the chorioid plexus of the human brain was found to contain vasopressin binding sites. In Alzheimer's disease a 2-fold increase in vasopressin binding sites was found in this structure (Korting et al., 1995). Vasopressin is thought to play a role in the choroid plexus with respect to ion and water transport and to reduce CSF production (Nilsson et al., 1992). An extension to the extrahypothalamic neurohypophysin containing fiber distribution in the human brain was given by Mai et al. (1993), but without a distinction of the fibers being vasopressinergic or oxytocinergic.

In the human PVN it is not possible to determine with certainty which neurons that express oxytocin or vasopressin are of a neuroendocrine nature and project to the neurohypophysis and which oxytocin neurons project to the brain where they act as neurotransmitter or neuromodulator. In the first place, unlike that of the rat, in the human PVN the cells cannot be subdivided into a part containing only magnocellular elements projecting to the neurohypophysis and one containing only parvicellular elements projecting to the brain stem, because there is a continuous distribution from small to large oxytocin and vasopressin neurons in the human PVN. Moreover, in contrast to the rat, neither type of oxytocin or vasopressin neuron of the human PVN is localized in a particular subnucleus of the PVN. This is also the case with corticotrophin-releasing hormone (CRH) neurons (Swaab et al., 1995; Raadsheer et al., 1993). The absence of an arrangement of the PVN into subnuclei is certainly not restricted to humans. It has also been observed in the cow, the cat and the guinea pig (Swaab et al., 1995).

The central vasopressinergic fibers may be involved in blood pressure and temperature regulation, regulation of osmolality and corticosteroid secretion and influence cognitive functions, aggres-

sion, social attachment and paternal behavior (Legros et al., 1980; De Wied and Van Ree, 1982; Buijs et al., 1983; Fliers et al., 1986; Legros and Ansseau, 1992; Holsboer et al., 1992; Insel, 1997). In contrast, oxytocin would give rise to sedation and lowering of blood pressure. Oxytocin levels rise after non-noxious stimulation such as touch, light exposure and high temperature. Oxytocin is held responsible for the antistress effects that occur during lactation and oxytocin has central effects on food intake as well (Uvnäs-Moberg, 1997). Oxytocin neurons are considered to be the putative satiety neurons for eating behavior. Our observations in Prader-Willi patients, who have an insatiable hunger and extreme obesity, support this idea, as a 42% decrease in oxytocin-expressing neurons in the PVN was found in this disorder (Figs. 5 and 6; Swaab et al., 1995). Increased oxytocinergic activity in depression may be related to eating disorders (Purba et al., 1996) and increased vasopressin levels to suicidal behavior (Inder et al., 1997). Alterations in PVN function have been proposed in schizophrenia too, on the basis of the highly decreased numbers of neurophysin-containing neurons that were found in the PVN of schizophrenic patients. No differences were found in the SON of schizophrenic patients, however (Mai et al., 1993), but it is not known if and how the PVN changes may contribute to the symptomatology of the disorder. In Parkinson's disease the number of oxytocin-expressing neurons in the hypothalamus is decreased (Purba et al., 1994).

Oxytocin, vasopressin and sexual behavior

Oxytocin is thought to be involved in affiliation, including pair bonding in monogamous animals (Insel, 1997) and maternal and reproductive behavior (Carter, 1992; Insel, 1992; Anderson-Hunt and Dennerstein, 1995). The synapses between vaso-

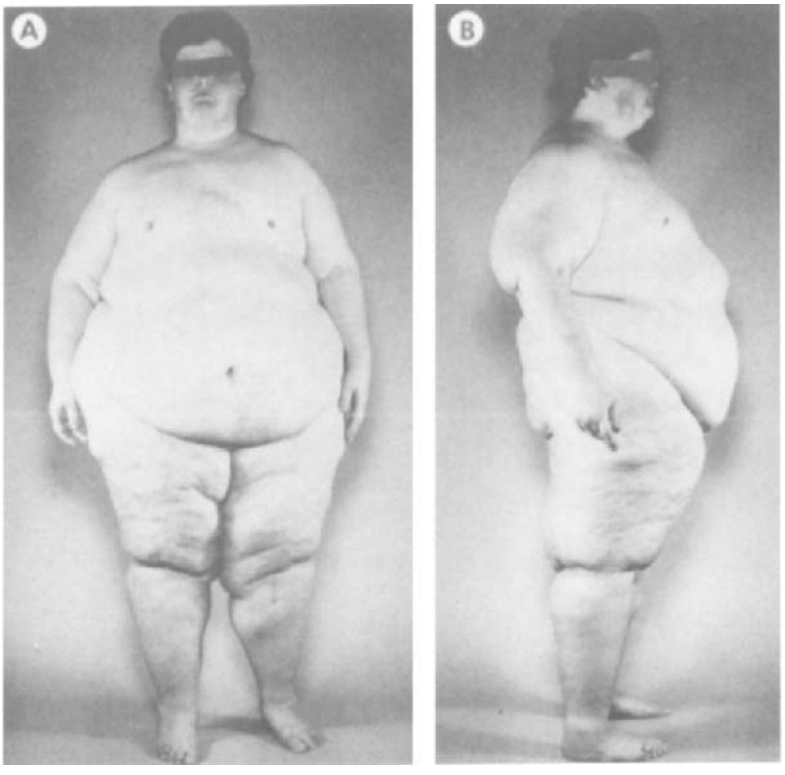


Fig. 5. Characteristic pattern of obesity in a patient with Prader-Willi syndrome (from Kaplan et al., 1991, with permission).

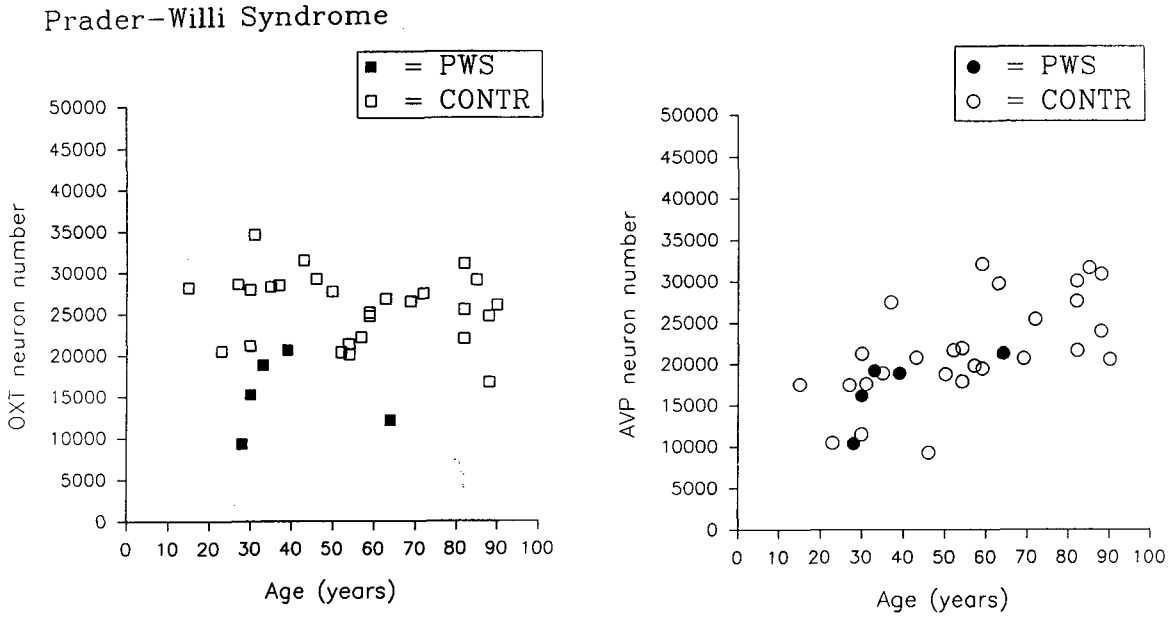


Fig. 6. Number of oxytocin-expressing (left panel) and vasopressin-expressing (right panel) neurons in the PVN of 27 controls and five Prader-Willi syndrome (PWS) patients. Note that the oxytocin neuron number of these patients is about half of that of the controls (left), which is not the case for vasopressin (right) (from Swaab et al., 1995, with permission).

pressin neurons of the SON and neuroendocrine LHRH neurons as observed in monkey suggest a coordinated role of vasopressin and gonadotropin secretion. In men, oxytocin might be involved in sexual arousal and ejaculation (Murphy et al., 1987) and in both men and women oxytocin induces contractions of smooth muscle cells and may thus facilitate transport of eggs and sperm (Carmichael et al., 1994). In female animals, oxytocin was found to facilitate estrus, sexual arousal, receptivity and other mating behaviors, including lordosis. An as yet unconfirmed case report has described a woman who began to take a contraceptive pill containing progesteron only. She experienced accentuated physiological and psychological sexual arousal after she had co-incidentally used a prescribed synthetic oxytocin spray for 'let-down' of breast milk (Anderson-Hunt, 1994; Anderson-Hunt and Dennerstein, 1995). In agreement with this case report, oxytocin levels are known to rise during sexual arousal and peak during orgasm in both women and men. In multiorgasmic subjects oxytocin peaks immediately prior to and during terminative orgasm, i.e. the oxytocin peak coincides with sexual saturation. The intensity of orgasmic contractions, but not their duration, correlated positively with increases in oxytocin levels. Naloxon decreases the level of pleasure at orgasm and blocks the periorgasmic rise of oxytocin levels (Murphy et al., 1990; Carmichael et al., 1994). Animal experiments show a mechanism of interaction between sex hormones and oxytocin by initiating the production of receptors for this peptide (Anderson-Hunt and Dennerstein, 1995). Indeed, oxytocin levels are significantly higher in women on oral contraceptives and oxytocin levels recorded during the menstrual week are significantly lower than during the rest of the period (Uvnäs-Moberg et al., 1989). The plasma concentration of vasopressin during the menstrual cycle is doubled on days 16–18 as compared to day 1 (Forsling et al., 1981). In the non-pregnant human uterus oxytocin and vasopressin receptors of the V_1 subtype are present (Guillon et al., 1987). Vasopressin may have a

role in stimulating uterine contractions. In this respect it is of interest that women with primary dysmenorrhea, who have an increased uterine activity and a decreased blood flow in the uterus have increased plasma levels of vasopressin (Åkerlund et al., 1979).

The fetal SON, PVN and birth

Fetal neurohypophysis and birth

> *... since the abnormal process of birth frequently produces no effect, difficult birth in itself in certain cases is merely a symptom of deeper effects that influenced the development of the fetus.*
>
> *Sigmund Freud, 1897*

Not only maternal but also fetal neurohypophysial hormones play an active role in the birth process. Fetal oxytocin was proposed to initiate parturition (Schriefer et al., 1982) or accelerate the course of labor (Swaab et al., 1977; Boer et al., 1980). Fetal vasopressin levels in umbilical cord blood are much higher following normal delivery than at any other stage of life (Chard et al., 1971; Oosterbaan and Swaab, 1989). Fetal vasopressin is one of the hormones that plays a role in the adaptation of the fetus to the stress of labor, for example by redistribution of the fetal blood flow with a marked reduction in the flow to gastrointestinal and peripheral circulations and an increase in the flow to essential organs such as the brain, the pituitary, the heart and the adrenals (Iwamoto et al., 1979; Pohjavuori and Fyhrquist, 1980). The neurons producing these neuropeptides are already present early in fetal life. Using neurophysin staining that did not distinguish between oxytocin or vasopressin neurons, Mai et al. (1997) detected staining SON neurons from 10 weeks of gestation onwards, roughly coinciding with the arrival of the optic tract fibers. Vasopressin and oxytocin have been found as early as 11 and 14 weeks of gestational age respectively (Fellmann et al., 1979; Burford and Robinson, 1982). Neurophysin was demonstrable at 13 weeks of gestation in the accessory nuclei and at 14 weeks of gestation in the PVN. Vasopressin-neurophysin was detected from 18 weeks of gestation onwards, and vasopressin-mRNA from 21 weeks of gestation in the SON, PVN and accessory nuclei (Murayama et al., 1993). An increase in vasopressin and oxytocin levels in the pituitary and the brain during the development of the fetus has been described by a number of researchers (Skowsky and Fisher, 1977; Schubert et al., 1981; Burford and Robinson, 1982; Khan-Dawood and Dawood, 1984).

Most cells of the magnocellular system seemed to derive from the region of the hypothalamic sulcus and migrate laterally and ventrally before expressing neurophysin at their site of settling. The cells possibly migrate along epidermal growth factor receptor positive radial glial cells that extend from the hypothalamic sulcus into the lateral hypothalamus. A dense catecholaminergic network of fibers is already present in the PVN of a 3- to 4-month-old fetus (Nobin and Björklund, 1973).

Because premature children are more sensitive to the stress of birth, we determined the number of oxytocin or vasopressin-expressing neurons in the human fetal hypothalamo-neurohypophysial system (HNS) in premature and at-term children. From the youngest fetus of our study onwards, so from a gestational age of 26 weeks, adult vasopressin and oxytocin cell numbers were found in both the SON and the PVN (Fig. 7; Wierda et al., 1991; Van der Woude et al., 1995). This is in agreement with the estimation of Dörner and Staudt (1972) that the hypothalamic nuclei are already completely formed around 25 weeks of gestation, on the basis of the disappearance of the matrix layer around the third ventricle at that age (Staudt and Stüber, 1977). The fetal HNS, however, is far from mature at term, in spite of the adult cell numbers. This is apparent, for example from the neuronal densities which are still decreasing after this period. Rinne et al. (1962) found a gradual increase in nuclear volume in the SON and PVN during fetal development, but he did not distinguish between oxytocin and vasopressin neurons. Judging by the strongly increasing nuclear size of the oxytocin neurons in the fetus during the last part of gestation (our unpublished data), these neurons seem to become gradually strongly activated towards term. This should, however, be confirmed by better measures of neuronal activity,

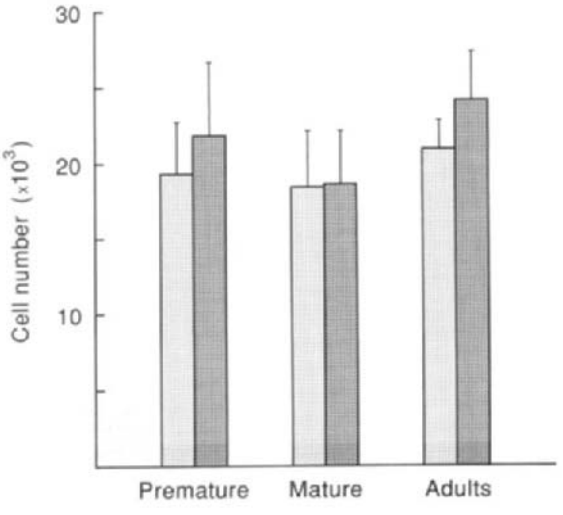


Fig. 7. Vasopressin (light bars) and oxytocin (dark bars) cell numbers in the PVN of premature (26–37 weeks) and mature (37–42 weeks) fetuses and in adults. Adult numbers were already present around 26 weeks gestation (from Goudsmit et al., 1992, with permission).

such as in situ hybridization for oxytocin-mRNA. So it seems quite possible that less mature oxytocin neurons in premature children would be, at least partly, responsible for the increased incidence of obstetrical problems. The idea of an active fetal role of oxytocin neurons in delivery is reinforced by a number of clinical observations. Firstly, human anencephalics do not have a neurohypophysis and have an impaired oxytocin and vasopressin release (Visser and Swaab, 1979; Oosterbaan and Swaab, 1987). In anencephalics expulsion takes twice as long and the birth of the placenta even takes three times longer, suggesting a role of fetal neuroendocrine mechanisms in speeding up the course of labor. In addition, the observation that about half of the anencephalics dies during the course of labor is a strong indication for the importance of an intact fetal brain to withstand the stress of birth (Honnebier and Swaab, 1973; Swaab et al., 1977). The second observation is derived from children suffering from Prader-Willi syndrome (see below). These children have considerable obstetrical problems (Wharton and Bresman, 1989), and we found that adult Prader-Willi patients have only 58% of the normal number of oxytocin neurons in adulthood but their number of vasopressin neurons is normal (Swaab et al., 1995). The third argument is based on the frequent perinatal problems found in septo-optic dysplasia (De Morsier, 1956) in which the fetal hypothalamo-neurohypophysial system is often damaged (Roessmann et al., 1987) (Fig. 8a,b). Moreover, prolonged labor and breech delivery have been documented in 50–60% of the idiopathic growth hormone-deficient children, a disorder that seems to be based on congenital hypothalamic pituitary abnormalities (Maghnie et al., 1991). The causality of the relationship between obstetric complications and neurological or psychiatric diseases such as schizophrenia (Geddes and Lawrie, 1995), might thus be quite the reverse from what is generally thought. A disturbed labor might be the first symptom of a brain disorder (see citation from Freud, before). How the fetal hypothalamus might play a role in fetal presentation is not known at present.

Immaturity at term may also affect the function of vasopressin neurons, though to a lesser degree than oxytocin neurons since the vasopressin neurons are already further advanced in the developmental process, something that has also been reported for other species. This is in agreement with the vasopressin levels in fetal cord, which are extremely high after delivery (Oosterbaan and Swaab, 1989). The adaptive vasopressin response (Pohjavuori and Fyhrquist, 1980) has been said to be induced by the stress of birth (Chard et al., 1971), by hypoxemia, acidemia (Daniel et al., 1983; Parboosingh et al., 1982) or by a rise in intracranial pressure associated with delivery. Perinatal hypoxia stimulates the vasopressin neurons in particular, as became apparent from their co-expression of tyrosine-hydroxylase (Panayotacopoulou et al., 1994). The physiological importance of neuroendocrine adaptive responses may also be deduced from the fact that two-thirds of the anencephalic children die during the course of labor (Honnebier and Swaab, 1973). These children do not have a functional neurohypophysis (Visser and Swaab, 1979). The observation that hereditary hypothalamic diabetes insipidus children from ditto mothers without vasopressin did not have a history of difficult labor at first sight seemed to suggest that the

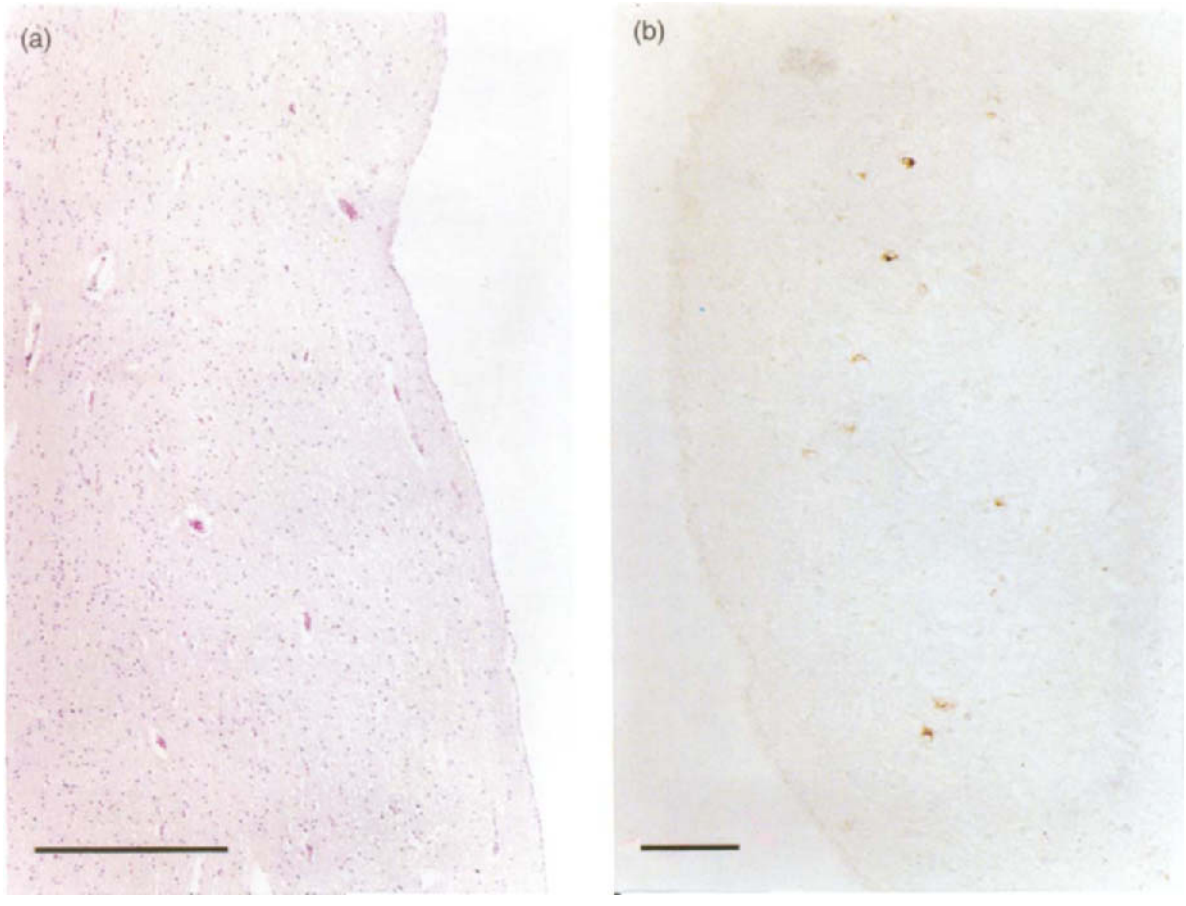


Fig. 8. Septo-optic dysplasia (De Morsier syndrome) in a girl of 4 years and 5 months. (A) The PVN is absent. Scale bar, 0.5 mm. (B) Only a few small PVN neurons were present that stained for the vasopressin precursor by means of an antiglycopeptide antibody (Boris Y-2). Scale bar, 100 μm.

absence of vasopressin alone does not seem to prevent an adequate neuroendocrine adaptive response of the fetus during labor (Swaab et al., 1982). However, later it became clear that vasopressin production in these children may be quite normal up to the age of 9 years or so (Bahnsen et al., 1992), so that these children do not give useful information on the putative involvement of fetal vasopressin.

Drinking disorders

As to the function of the posterior lobe, the experimental evidence is unequivocal. Its removal causes no symptoms. Moreover, its structure is non-glandular. Camus and Roussy were quite justified in speaking of it as an atrophied nervous lobe.... We have, therefore, no evidence that pituitrin is anything more than a pharmacologically very interesting extract
(Bailey and Bremer, 1921 cited by Anderson and Haymaker, 1974).

Pathology of the neurohypophysis

The neurohypophysis consists of (1) the pars nervosa (neural or posterior lobe), (2) the infundibular process (pituitary stalk), that contains the nerve tracts from the supraoptic and paraventricular

nucleus (SON and PVN), a thin tongue of anterior pituitary tissue (pars tuberalis containing predominantly gonadotrophs) and the vessels of the portal system and (3) the pars infundibularis (infundibulum). Clinically, pathology of the neurohypophysis may lead to diabetes insipidus or to inappropriate secretion of vasopressin (Schwartz–Bartter syndrome). Apart from disturbances of water metabolism, abnormalities in the posterior pituitary, particularly space-occupying lesions, may cause symptoms such as headache and visual disturbances. In addition, the intracranial pressure may increase, producing anterior-pituitary compression. Damage to the pituitary stalk may interrupt the portal circulation and lead to infarction of the anterior lobe and thus to endocrine impairments. Congenital malformations in the neurohypophysis have also been described, such as persistence of the infundibular recess by which the third ventricle is protruding into the neurohypophysis. The infundibular recess in the neurohypophysis normally disappears in human embryos by the 45 mm stage (Cabanes, 1978). Moreover, duplication of the pituitary, of the adenohypophysis as well as the neurohypophysis have been described (Hori, 1983). In addition, loss of the infundibulum or pituitary stalk due to traumatic damage has been reported (Grossman and Sanfield, 1994). Dystopia of the neurohypophysis may either be asymptomatic or go together with anterior pituitary anomalies (Aydin and Ghatak, 1994) In the empty-sella syndrome the intrasellar CSF does not seem to influence posterior lobe function (Zucchini et al., 1995). No patients have been reported with isolated diabetes insipidus or other hypothalamic-pituitary disorders who also had an empty sella (Cacciari et al., 1994).

The following histopathological phenomena may be found in the neurohypophysis (for reviews see Kovacs, 1984; Treip, 1992; Horvath et al., 1997):

- Basophilic ACTH and βMSH containing cell invasion (Osamura and Watanabe, 1978) is generally not present before the age of 20 years. This phenomenon does not occur before puberty and is frequently (in 30%) seen in older subjects, especially in aging men. It is generally not related to any endocrine abnormality (Sheehan and Kovacs, 1982; Sano et al., 1993). However, some authors consider these cells to be one of the possible origins of basophil pituitary adenomas. In the literature two cases of basophil adenomas in the posterior lobe have indeed been recorded (Kuebber et al., 1990).
- Glandular structures (Rasmussen, 1933), i.e. ectopic salivary gland tissue resembling serous acinar and duct cells (Schochet et al., 1974; Osamura and Watanabe, 1978; Horvath et al., 1997) and lymphocytic foci (Shanklin, 1951) are common incidental findings in the posterior lobe or stalk without clinical consequences. Chronic inflammation with infiltration of lymphocytes, predominantly of the T cell and CD4+ type, and plasma cells are found in the case of lymphocytic infundibulo-neurohypophysitis and may cause diabetes insipidus. In the case of lymphocytic infundibulo-neurohypophysitis, thickening of the pituitary stalk, enlargement of the neurohypophysis and absence of the hyperintense MRI signal of the posterior pituitary are found.
- The commonest finding in the infundibular stem and process is acute haemorrhage, often petechial, but sometimes large enough to cause appreciable damage to the infundibular process. Necrosis within the infundibular process itself is very rare. Haemorrhages and necroses may be associated with traumatic head injuries, postpartum necrosis of the anterior pituitary, increased cranial pressure, shock, disseminated intravascular coagulation, septicemia, and various hematological disorders. Accumulation of neurosecretory material and retraction bulbs are evidence of ruptured axons. In the case of thrombotic thrombocytopenic purpura, vessels in the neural lobe may contain hyaline thrombi. Agonal thrombi may also be seen in the vessels of the infundibular stem.
- Chronic changes following lesions are atrophy and loss of pituicytes. Haemosiderin deposition may be found in longstanding cases and can appear within 8 days of injury.
- Cystic changes in the infundibular process have been described.
- Hypovolaemic shock of the mother at the time of delivery may not only cause pituitary necrosis, but may also affect the tuber cinereum, pituitary stalk, SON and PVN. Hypopituitarism of pregnancy may

be accompanied by diabetes insipidus of sudden onset following severe postpartum haemorrhage. However, true diabetes insipidus is rare in Sheehan's syndrome, and lesions may be present in the posterior lobe without corresponding clinical symptoms. Probably the amount of damage to the SON and PVN will determine whether or not diabetes insipidus will occur, but other factors are certainly not excluded (Sheehan and Kovacs, 1982). And indeed, in cases of postpartum hypopituitarism considerable degeneration in the SON and PVN has been reported. The estimated numbers of SON neurons remaining was often only some 20–25%. In some cases the accessory SON may disappear as well. The PVN is sometimes as strongly affected as the SON, but sometimes retains its normal neuronal numbers. In addition, nearly all cases had petechial haemorrhages in the hypothalamus, apparently related to the terminal coma (Whitehead, 1963).

- Granulomas may be present on the basis of tuberculosis, neurosarcoidosis, syphilis or Wegener's granulomatosis. The neurohypophysis is also frequently involved in histiocytosis. Diabetes insipidus may be an early sign of this disease (Catalina et al., 1995). On MRI a thickened stalk may be seen as expression of preclinical histiocytosis (Zucchini et al., 1995). Numerous eosinophilic leukocytes and lipid-laden foamy macrophages may be present or fibrosis may prevail.
- Infections and fibrosis may also be found in the neurohypophysis. Patients with septicaemia sometimes have foci of leucocytes in the posterior lobe.
- Granular cell tumors (termed choristomas by Sternberg in 1921), and granular cell myoblastomas or tumorettes by Shanklin in 1947) are the most common primary neurohypophysial tumors. They occur in some 5–17% of the pituitaries (Shanklin, 1953; Sano et al., 1993) and are found after the second decade in some 6% of the pituitary glands (Luse and Kernohan, 1955). They consist of large cells with granular, lightly eosinophylic cytoplasm (Fig. 9). They are mostly small, 1–2 mm or even smaller, and are not evident on gross inspection. They grow slowly, are histologically benign, well demarcated, but unencapsulated. The majority remains asymptomatic. In those rare cases that granule cell tumors are symptomatic they may give diabetes insipidus, hypopituitarism, visual impairment or headache (Symon et al., 1971; Massie, 1979; Barrande et al., 1995; Ji et al., 1995). Histologically, granular cell tumors are composed of loosely apposed, large, spherical, oval or polygonal cells with eccentrically located nuclei and abundant acidophilic cytoplasm, containing PAS positive neuramic acid and carbohydrate containing granula that appear to be lysosomes under the EM. The brown cytoplasmatic granules (Fig. 9) may be sufficiently numerous to import a brown pigmented appearance to the tumor (Massie, 1979). The cells probably originate from pituicytes (Luse and Kernohan, 1955; Jenevein, 1964; Massie, 1979; Müller et al., 1980; Horvath et al., 1997). Granular cell tumors label with lectin,

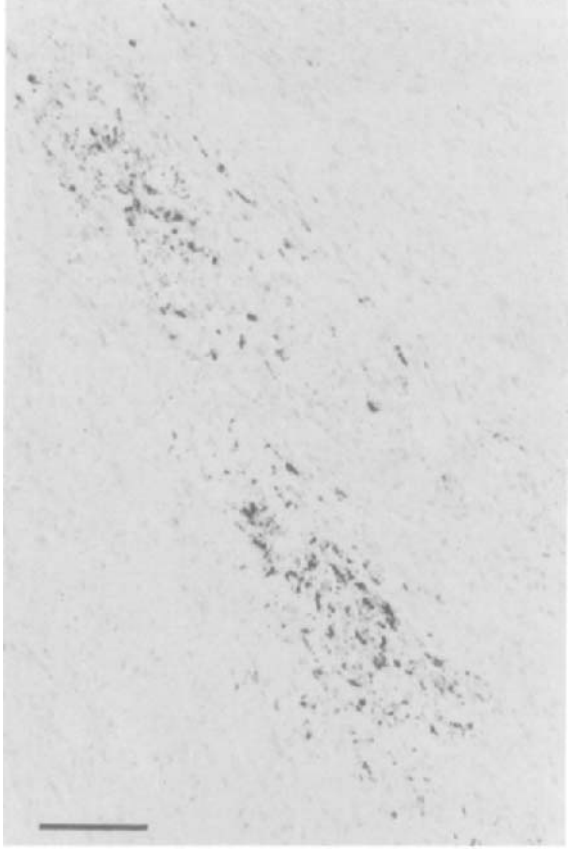


Fig. 9. Cells of a granular cell tumor (choristoma) in the neurohypophysis. Note the brown pigmented cytoplasmatic granules (NHB # 92-001). Scale bar, 100 μm.

S-100 and not with neuron-specific enolase, myelin basic protein, vimentin, keratin or desmin and mostly not with GFAP. Some showed reactivity, however, for α-1-antitrypsin, α-1-antichymotrypsin and cathepsin B. The latter marker suggests a lysosomal disorder. The fact that GFAP is usually negative does not support the pituicyte as a progenitor of granular cell tumors, but it also does not exclude this possibility (Nishioka et al., 1991). Indeed, Barrande et al. (1995) and Lafitte et al. (1994) both reported a granular cell tumor to be positive for GFAP.

- Metastatic carcinomas in the posterior pituitary constitute the most important neoplasms. They may be derived from, e.g. carcinomas of the bronchus, breast, colon, prostate, malignant melanoma, sarcomas, lymphoma, Hodgkin's disease, or leukemia and may give rise to diabetes insipidus.
- Only seldom are other neoplasms found in the neurohypophysis, i.e. gliomas that are only rarely derived from pituicytes. A few cases of pilocytic pituitary astrocytomas or pituicytomas have been described. MRI shows extension of the tumor into the pituitary stalk. Panhypopituitarism may be an early manifestation of this tumor, but diabetes insipidus may be absent, suggesting vasopressin release to take place above the level of the tumor (Nishizawa et al., 1997). In addition, gangliogliomas, hamartomas (Fig. 10), epidermoids, suprasellar germinomas, craniopharyngiomas and lipomas have been reported (Hurley et al., 1994). The first abnormal MRI sign in case of germinoma in children and adolescents was pituitary stalk thickening (Mootha et al., 1997). One case of a meningioma of the pituitary stalk has been described that must have originated from the arachnoid membrane since it had no dura attachment (Hayashi et al., 1997). A few cases of gangliocytomas have also been reported. Neoplasms of the infundibulum are described under various names, i.e. infundibuloma, hamartoma, glioma or astrocytoma.

An early stage of cytoskeletal alterations as revealed by antibodies against abnormally phosphorylated tau (e.g. AT8, PHF-1 and Alz-50) are found in fibers and Herring-body-like swellings in

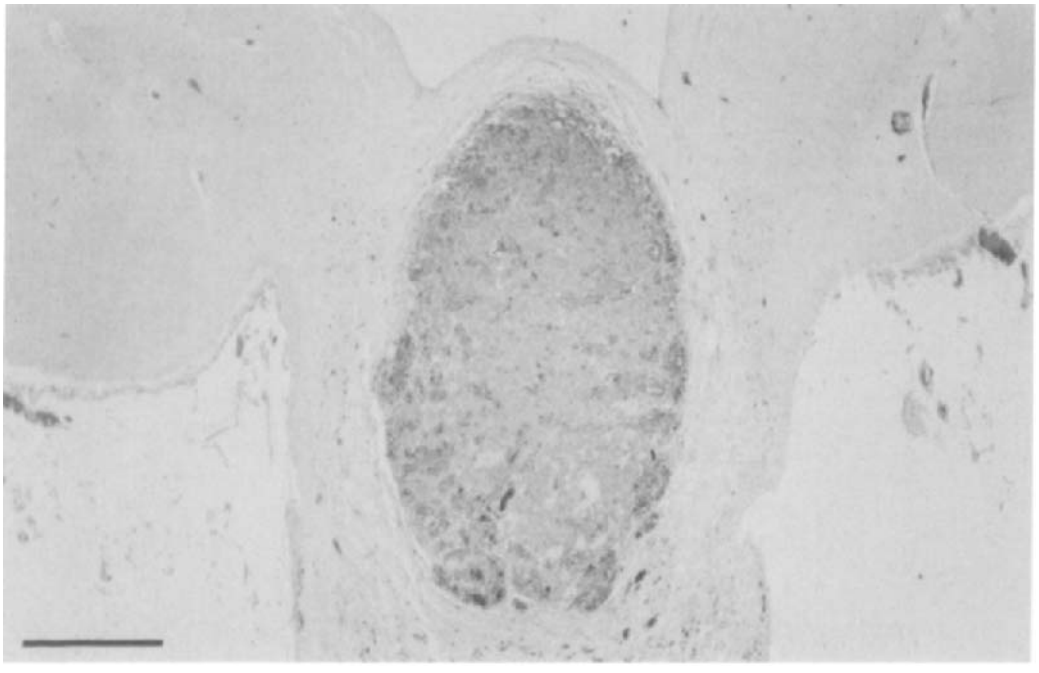


Fig. 10. Asymptomatic hypothalamic hamartoma (NHB # 84186; 29 years of age) in the median eminence/pituitary stalk region stained for its strong vasopressin innervation. The tumor was less densely innervated by oxytocin fibers and did not stain for LHRH. Scale bar, 1 mm.

some aged patients, even if the brain was devoid of Alzheimer changes. Such changes are difficult to detect with the sensitive silver method. The relationship between these early Alzheimer changes in the neurohypophysis and those in the SON, PVN and the rest of the brain should be further investigated. In addition, the functional implications of these cytoskeletal changes are not yet clear (Schultz et al., 1997).

Pathological states of the neurohypophysis may be reflected in changes in the MRI high intensity signal of the posterior pituitary that is normally present in 90% of the subjects (Brooks et al., 1989; Tien et al., 1991). It was first thought to be caused by fat within the sella turcica, but the source of the hyperintense MRI signal is now presumed to be the neurosecretory granules containing vasopressin (Fujisawa et al., 1989). No attention has been paid so far to the contribution of oxytocin-containing granules. It should be noted that also in the case of persistent elevation of vasopressin plasma levels as found in diabetes mellitus and hemodialysis, the hyperintense MRI signal in the neurohypophysis may be decreased, possibly due to depletion of vasopressin storage. When treating for hyperglycaemia, plasma levels of vasopressin promptly decrease and the hyperintense MRI signal reappears within 1–2 months (Fujisawa et al., 1996; Sato et al., 1995). The high intensity MRI signal is frequently absent in the case of macroadenoma of the anterior pituitary, craniopharyngioma, traumatic stalk transsection, patients with empty sella syndrome, and diabetes insipidus. In both primary idiopathic diabetes insipidus and secondary diabetes insipidus due to e.g. germinoma, teratoma (Mootha et al., 1997), Wolfram syndrome or histiocytosis-x, the normal high intensity MRI signal was not detected. In some patients with macroadenomas, a small, high signal intensity region was seen above the pituitary gland without any high intensity from within the gland itself (Colombo et al., 1987; Fujisawa et al., 1987b), suggesting the presence of a newly formed 'miniature-posterior lobe' above the level of the neurohypophysis.

Diabetes insipidus

> *The brain secretes thoughts as the kidney secretes urine.*
>
> *Jakob Moleschott (1822–1893)*

Diabetes insipidus is characterized clinically by polyuria and polydipsia and may be due to vasopressin deficiency, a lack of kidney receptors for vasopressin or primary polydipsia.

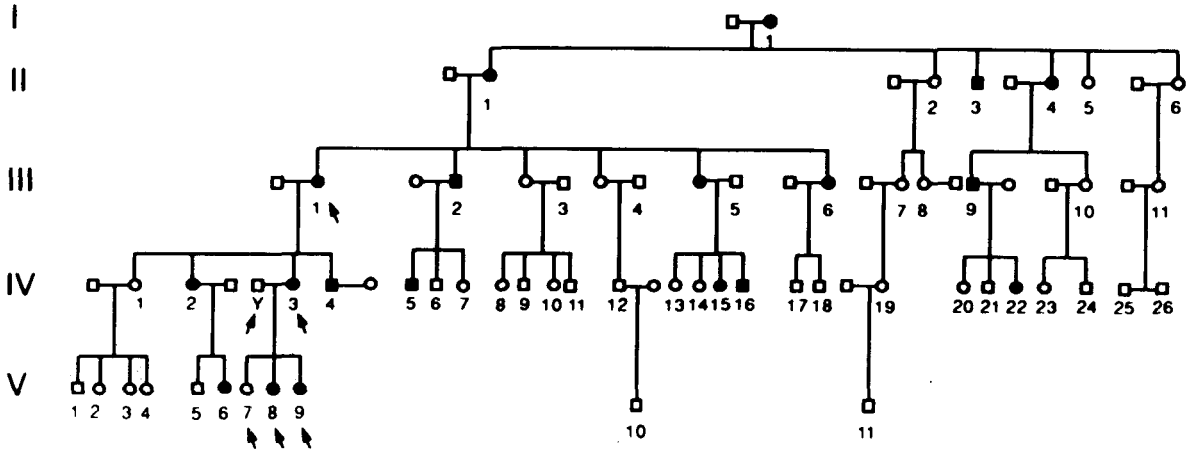


Fig. 11. Pedigree of the Dutch hereditary hypothalamic diabetes insipidus family comprising five generations. Black symbols denote affected individuals. Females are indicated by circles and males by squares. Samples were available from individuals marked by the arrows (from Bahnsen et al., 1992, with permission).

Familial diabetes insipidus

Familial hypothalamic diabetes insipidus is transmitted as an autosomal dominant gene. Affected individuals have low or undetectable levels of circulating vasopressin and suffer from polydipsia and polyuria, and they respond to substitution therapy with exogenous vasopressin or analogues. Urine production may amount to some 20 litres per day. Members of a Dutch family suffering from this disease (Fig. 11) appeared to have a point mutation in one allele of the affected gene, based upon a G to T transversion at position 17 of the neurophysin encoding exon B on chromosome 20 (Bahnsen et al., 1992) (Fig. 12). In a Japanese diabetes insipidus family a G to A transition has been described in the same exon (Ito et al., 1991). A number of other mutations have now been found in familial hypothalamic diabetes insipidus (Fig. 13), i.e. two missense mutations that altered the cleavage region of the signal peptide, seven missense mutations in axon 2, one nonsense mutation in axon 2 and 3 nonsense mutations in axon 3 (Miller, 1993; Yuasa et al., 1993; Nagasaki et al., 1995; Rittig et al., 1996). Raug et al. (1996) found a point mutation in the carboxy-terminal domain of neurophysin II. Both in primary idiopathic diabetes insipidus and secondary diabetes insipidus, e.g. due to germinomas, teratoma, Langerhans cell histiocytosis, or Wolfram syndrome, the normal high MRI signal of the posterior lobe is generally not detected (Fujisawa et al., 1987b; Tien et al., 1991; Rutishauser et al., 1996), although there seem to be exceptions to this rule (Miyamoto et al., 1991; Maghnie et al., 1992, 1997). The source of the hyperintense MRI signal of the posterior pituitary is most probably the neurosecretory granules that contain vasopressin (Fujisawa et al., 1989). It has, however, so far not been studied what the contribution of oxytocin and its precursor might be to the bright spot. Increased release due to persistent elevation of plasma vasopressin levels, as found in diabetes mellitus, may also go together with a decreased MRI signal intensity of the neurohypophysis (Fujisawa et al., 1996). The presence of a bright spot in diabetes insipidus may thus depend on the type of mutation, the turnover of neuropeptides in the neurohypophysis and the amount of oxytocin.

The few available postmortem histological observations in families with hereditary hypothalamic diabetes insipidus point to severe neuronal

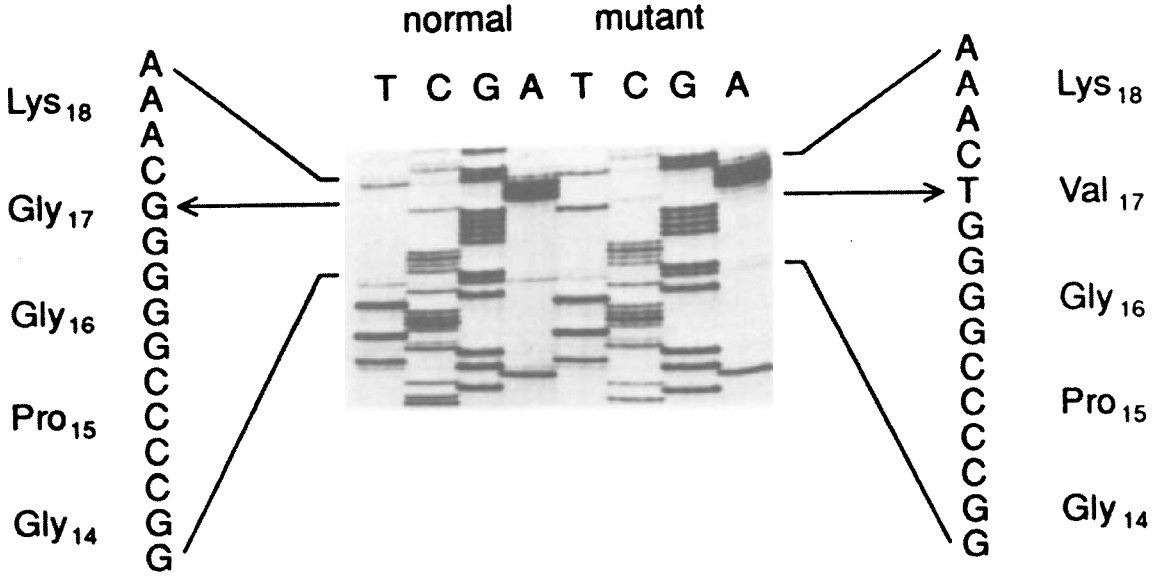


Fig. 12. DNA sequencing gel demonstrating the difference in exon B between the normal and the mutated vasopressin-neurophysin gene allele of the individual IV-3 (see Fig. 11). The missense mutation G-T is indicated by arrows. Numbering of the deduced amino acid sequence corresponds to human neurophysin (from Bahnsen et al., 1992, with permission).

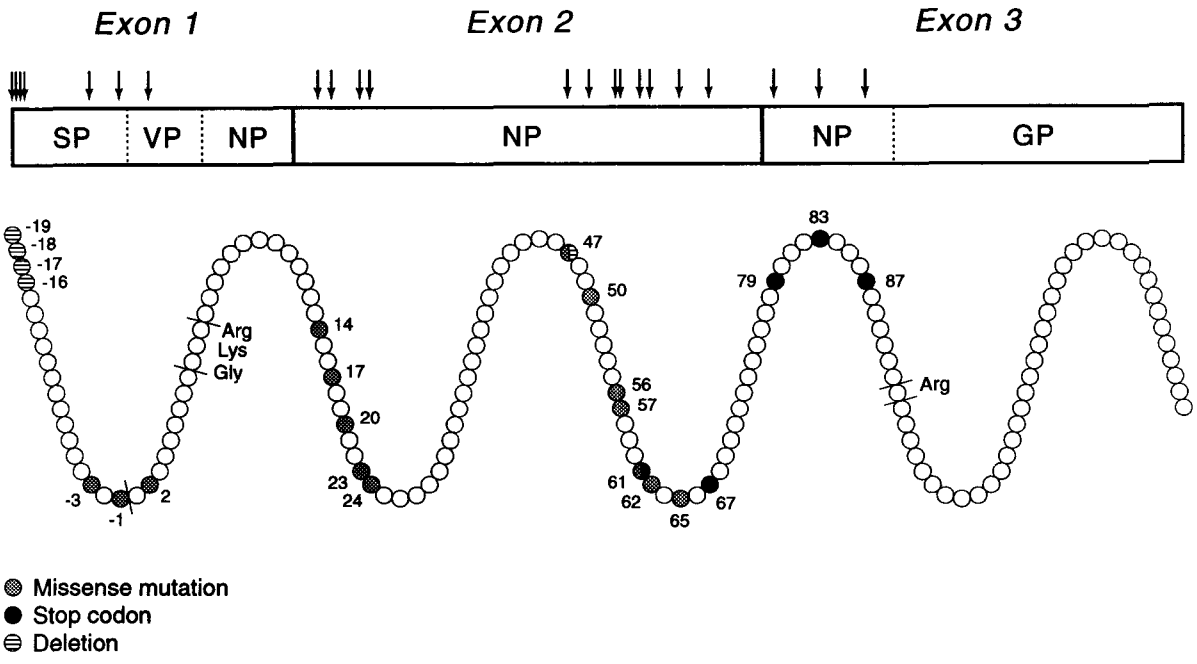


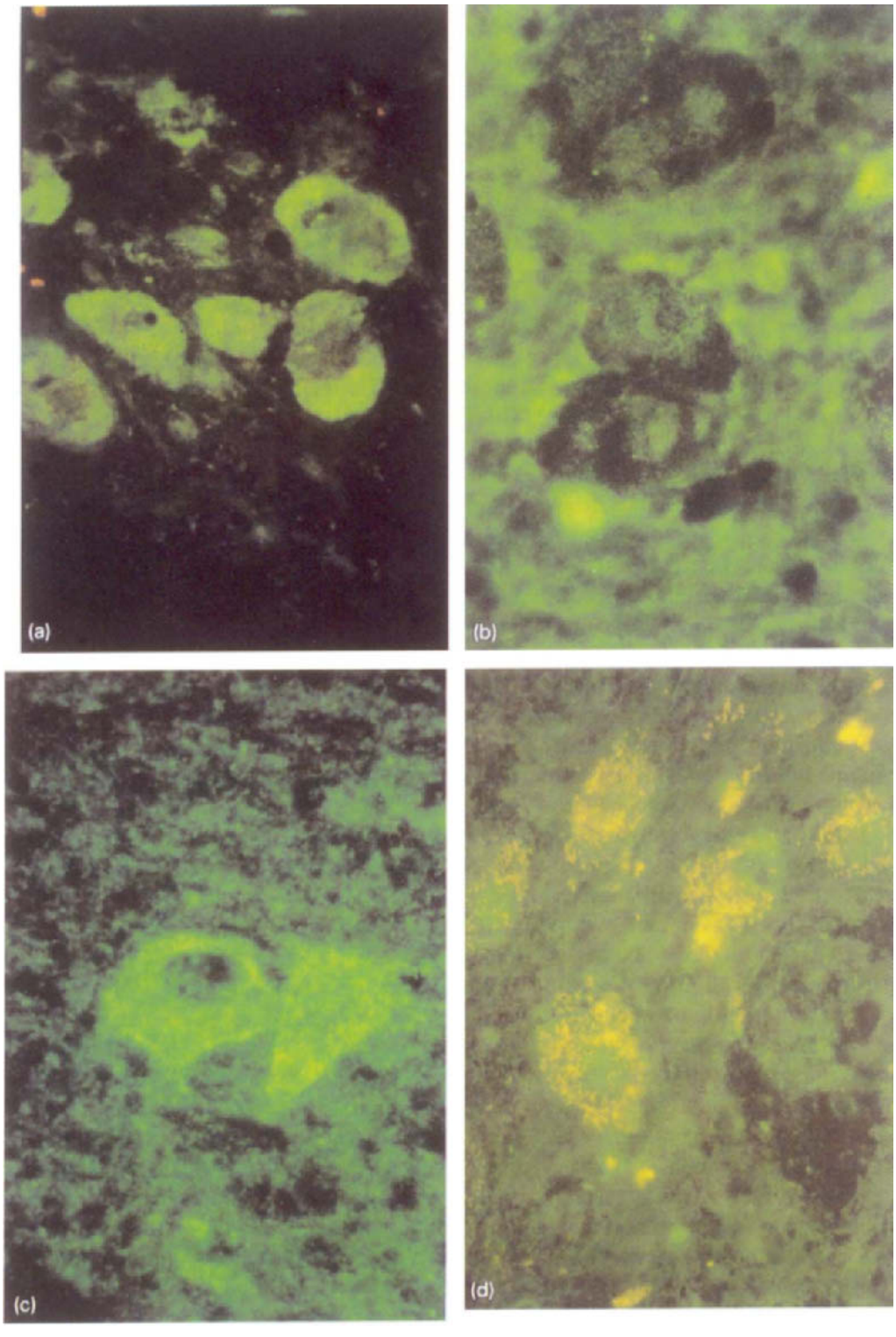
Fig. 13. Schematic diagram of the coding regions of the vasopressin (AVP)-neurophysin (NP) gene containing the glycopeptide (GP) moiety and the primary structure of the preprohormone, showing location and type of mutations identified in familial hypothalamic diabetes insipidus (from Rittig et al., 1996 with permission).

death in the SON and PVN associated with a loss of nerve fibers in the posterior pituitary (Hanhart, 1940; Braverman et al., 1965; Green et al., 1967; Bergeron et al., 1991) suggesting that the mutated product might be toxic to the neurosecretory cell. Slowly acting toxicity would also explain the variable age at onset of the disease (Schmale et al., 1993) such as in our observations that diabetes insipidus did not strike until an individual reached the age of approximately 9 years (Bahnsen et al., 1992). Other observations, too, indicate that vasopressin secretion is normal for the first few years of life, but that diabetes insipidus then develops rapidly, after which it may continue to aggravate slowly for a decade or more (McLeod et al., 1993). In one case, a 44-year-old man with hereditary diabetes insipidus, no clear cell death was found in the SON and PVN, but immunocytochemically the nuclei hardly stained for vasopressin in the PVN (Nagai et al., 1984). One can perhaps consider this as an early phase of degeneration.

When the mutant of the Dutch kindred was stably expressed in a mouse pituitary cell line, the mutant precursor was synthetized, but processing and secretion were dramatically reduced and the protein did not seem to reach the trans-Golgi network (Olias et al., 1996). Studies in which various other human mutant vasopressin precursors were expressed in cell lines also showed an accumulation of the mutated vasopressin precursor in the endoplasmic reticulum and a reduced viability of the cells (Ito et al., 1997). This may thus be the mechanism that causes the neurosecretory neurons in hereditary hypothalamic diabetes insipidus to degenerate.

Autoimmune diabetes insipidus

Recently an autoimmune form of hypothalamic diabetes insipidus has been described with circulating autoantibodies against the vasopressin cell surface (Fig. 14). Such autoantibodies could not be demonstrated in hereditary forms of diabetes insipidus. It has not yet been established whether the autoantibodies observed in diabetes insipidus


(a)
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are indeed cytotoxic and might destruct the vasopressin cell bodies (Scherbaum, 1992), but the presence of such antibodies might go together with partial diabetes insipidus, possibly even for a long period of time (De Bellis et al., 1994). In addition, a case of diabetes insipidus caused by nonspecific chronic inflammation of the hypothalamus was reported. As acute multifocal placoid pigment epitheliopathy with an immunogenic predisposition was found, and human leucocytic antigen class I antigen A2 and class II antigen DR4 were observed it might be a case of autoimmune reaction (Watanabe et al., 1994). In patients with idiopathic diabetes insipidus, thickening of the pituitary stalk, enlargement of the neurohypophysis and absence of the hyperintense MRI signal were found. Biopsies of the neurohypophysis or pituitary stalk revealed chronic inflammation with infiltration of lymphocytes predominantly of the CD4+ subpopulation and plasma cells. The natural course of the lymphocytic infundibulo-neurohypophysitis generally seems to be self-limited (Imura et al., 1993; Koshiyama et al., 1994; Paja et al., 1994). However, cases with necrotizing infundibulo-hypophysitis and a combination of diabetes insipidus and hypopituitarism have been described. It is possible that this disorder as described by Ahmed et al. (1993) represents an end stage of lymphocytic infundibulitis, but alternatively it may be a unique syndrome. Wegener's granulomatosis is a systematic necrotisizing vasculitis with neurological symptoms in some 50% of the cases. Anti-neutrophil cytoplasmic antibodies are present. A few cases have been described in which the disease remained confined to the anterior and posterior pituitary, causing hypopituitarism and diabetes insipidus. The patients did not respond to immunosuppressive therapy with cyclophosphamide but in some patients corticosteroids gave a clinical remission (Roberts et al., 1995; Rosete et al., 1991). In addition, it should be noted that iatrogenic antibodies may be raised by treatment with vasopressin or pituitary snuff.

Pregnancy induced diabetes insipidus

During pregnancy a transient form of diabetes insipidus sometimes occurs. This may be partly due to the presence of vasopressinase during pregnancy. Vasopressinase of pregnancy is the same enzyme as cystine-aminopeptidase (CAP) or oxytocinase, and is produced by the placenta. It may, at least partly, be responsible for the fact that the metabolic clearance of vasopressin during pregnancy increases 4-fold. The enzyme decreases to undetectable levels in several days postpartum. Cases have been described with a transient diabetes insipidus during pregnancy due to extraordinary high plasma CAP. However, a more likely cause of polydipsia and polyuria during pregnancy is the unmasking of subclinical forms of either central or nephrogenic diabetes insipidus. Apart from the presence of CAP, a decreased threshold for thirst contributes to the aggravation of diabetes insipidus. When, during pregnancy, thirst increases, more water is consumed and the urine volume becomes unacceptable. The lowering of the thirst threshold is accompanied by a similar lowering of the osmotic threshold for the release of vasopressin. The

Fig. 14. (a) Autoantibodies to hypothalamic vasopressin cells. An unfixed 7 μm cryostat section of human hypothalamus at the level of the supraoptic nucleus (SON) was incubated with native serum from a patient with idiopathic hypothalamic diabetes insipidus and stained with FITC-labeled anti-human-IgG. Note that the cytoplasm of large cells is stained. It could be shown by the four-layer double-fluorochrome immunofluorescence test with anti-vasopressin in the second sandwich that vasopressin cells were stained (magnification: ×250). (b) The same area of the SON as in (a) incubated with normal human serum and FITC-labeled polyvalent anti-human immunoglobulin. Note that the background is brighter than the dark neurosecretory cell bodies (magnification: ×400). (c) The same area of the SON as in (a) incubated with the serum of a patient with systemic lupus erythematosus containing the rare anti-ribosomal antibodies visualized by FITC-labeled anti-human IgG which may in a very rare case disturb the detection of vasopressin cell antibodies. Note the coarsely granulated cytoplasmic staining of the two large cell bodies (magnification: ×400). (d) A cryostat section (7 μm) of human hypothalamus at the level of the SON. The specimen was obtained from a donor aged 50 years. The section was incubated with normal human serum and FITC-labeled polyvalent anti-human immunoglobulin. The autofluorescent lipofuscin deposits in the cell bodies of large neurosecretory cells hamper the evaluation of test results (magnification: ×250) (from Scherbaum, 1992, with permission).

response is that pregnant women drink more water that is subsequently retained. The mechanism for the altered osmoregulation in pregnancy is obscure, but the combination of changes in water homeostasis and vasopressin metabolism that occurs in normal pregnancy seems to provide an explanation for the transient expression of diabetes insipidus in pregnant women, especially in case of latent forms of neurogenic or nephrogenic diabetes insipidus (Durr et al., 1987; Iwasaki et al., 1991; Robinson and Amico, 1991; Treip, 1992; Williams et al., 1993; Van der Post et al., 1994; Lindheimer and Davison, 1995; Naruki et al., 1996).

Other causes of diabetes insipidus

Diabetes insipidus has also been observed as part of a midline developmental anomaly, e.g. in septo-optic dysplasia (Fig. 8), in dystopia of the neurohypophysis, in hypothalamic Langerhans cell histiocytosis, following skull fracture, hypoxic/ischaemic brain damage, hemorrhage, infarcts, inflammation and abscess formation, tumors of the hypothalamus, including metastases, following traumatic injuries and after surgical manipulations affecting either the SON and PVN or, more frequently, following sectioning of the hypothalamo-neurohypophysial tract. Lesions of the neurohypophysis alone may not result in diabetes insipidus when the neurosecretory nuclei remain intact. In addition, diabetes insipidus may occur in neurosarcoidosis (Treip, 1970; Rudelli and Deck, 1979; Stern et al., 1985; Fujisawa et al., 1987b; Bell, 1991; Laing et al., 1991; Arisaka et al., 1992; Catalina et al., 1995). A syndrome of partial diabetes insipidus has been reported in anorexia nervosa, where vasopressin seems to be secreted erratically, independent of plasma sodium levels (Gold et al., 1983). Structural lesions of the hypothalamus in children may go together with weight gain, diabetes insipidus, osmoreceptor dysfunction (hypernatraemia with absent thirst), pituitary deficiencies and hyperprolactinaemia (Cianfarani et al., 1993).

The hypothalamic forms of diabetes insipidus should be distinguished from a molecular defect in the vasopressin V2-receptor gene causing nephrogenic diabetes insipidus, a rare X-linked disease (Bichet et al., 1988; Holtzman et al., 1993). It is characterized by normal or elevated concentrations of vasopressin and an insensitivity to exogenously administered vasopressin analogues. In masked diabetes insipidus, polyuria in nephrogenic diabetes insipidus is completely concealed by concomittant adrenal failure. Polyuria immediately starts following corticosteroid replacement, probably based on an intrarenal mechanism. Cortisol seems to be required for an optimal excretion of water (Iwasaki et al., 1997). On the other hand, we observed a strongly decreased vasopressin staining in the SON and PVN following corticosteroid treatment (Erkut et al., 1998), indicating a lack of free vasopressin that may also contribute to unmask diabetes insipidus.

Wolfram syndrome (acronym: DIDMOAD: diabetes insipidus, diabetes mellitus, optic atrophy and deafness; MIM222300) was first described by Wolfram and Wagener (1938). We found in the two Wolfram patients with diabetes insipidus, that the SON contained hardly any neurosecretory neurons in a thionin staining and no neurons that stained processed vasopressin. The PVN did not contain vasopressin-expressing neurons either (Fig. 15). However, using a potent antibody against the vasopressin precursor (anti-glycopeptide 22-39 Boris Y-2) a considerable number of vasopressinergic neurons was present in the PVN, although the cells were clearly too small (Fig. 15). No vasopressinergic neurons were found in the SON with Boris Y-2. The oxytocin expression in the SON and PVN seemed normal. In the two Wolfram patients who did not have vasopressin staining there was also an absence of protein convertase (PC)-2 and the molecular chaperone 7B2, strongly suggesting the presence of a processing disturbance of the vasopressin precursor (Gabreëls et al., 1998).

Lesion of the pituitary stalk

Following surgical hypophysectomy or pituitary stalk sectioning, the SON and PVN show a marked nerve cell loss that is usually more pronounced in the SON than in the PVN (Daniel and Prichard, 1975) (Figs. 16 and 17). Similar changes are found in the SON and PVN when the stalk is interrupted by metastasis (Duchen, 1966). Pituitary stalk sectioning has been employed to inhibit hypothalamic stimulation of the pituitary, e.g. in the treat-

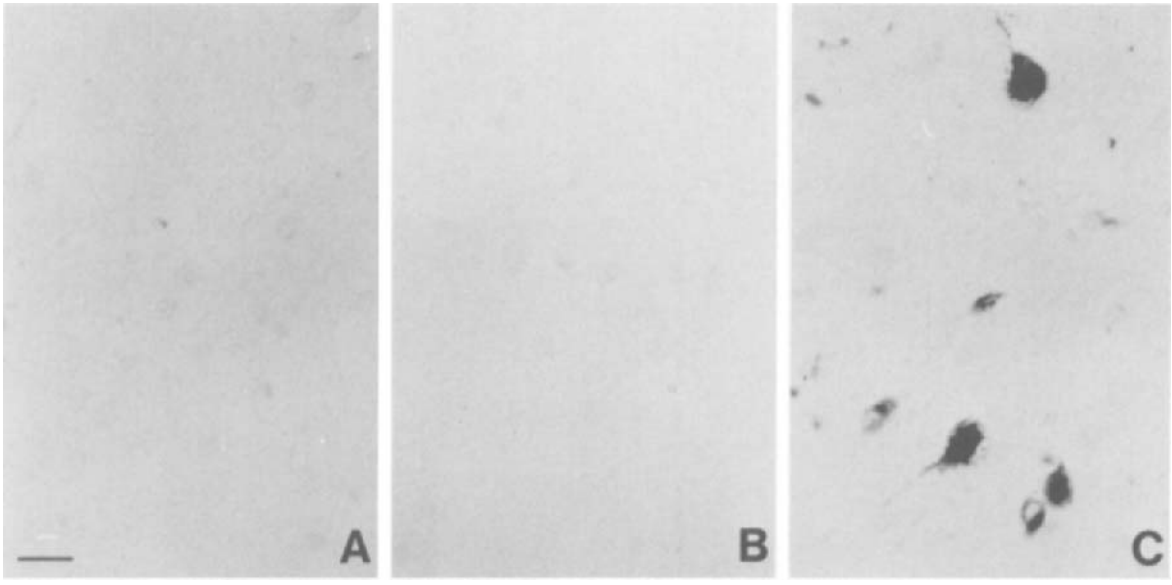

Fig. 15. Paraffin sections through the paraventricular nucleus (PVN) of a Wolfram syndrome patient # 94-133 (A) with the antibody #III-D-7 that recognizes processed vasopressin no immunoreactivity is found. (B) No immunoreactivity is present either with the antibody # PS41 predominantly recognizing the processed form of NP, but (C) many positive cells are stained with the antibody Boris Y-2 recognizing the glycopeptide part of the VP precursor. These data indicate a processing disorder. Scale bar, 25 μm (from Gabreëls et al., 1998, with permission).

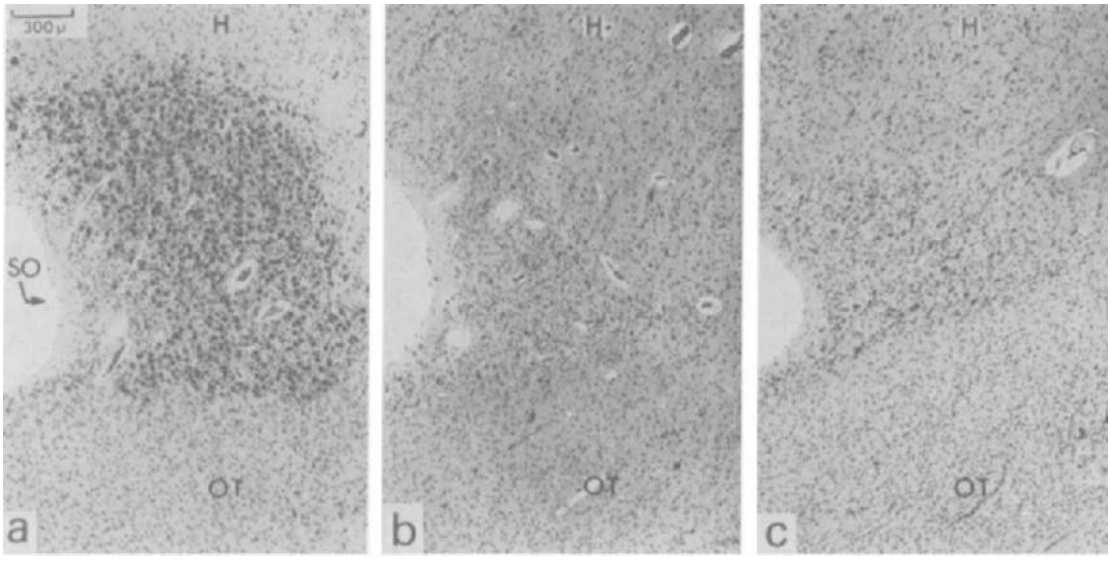

Fig. 16. Effect of hypophysectomy on the SON. Nissl stained sections through the SON of a normal subject (a), and of two patients who had been hypophysectomized for 4 months (b) and 8 years (c). Note the great loss of nerve cells in the nucleus after hypophysectomy (from Daniel and Prichard, 1975, with permission).

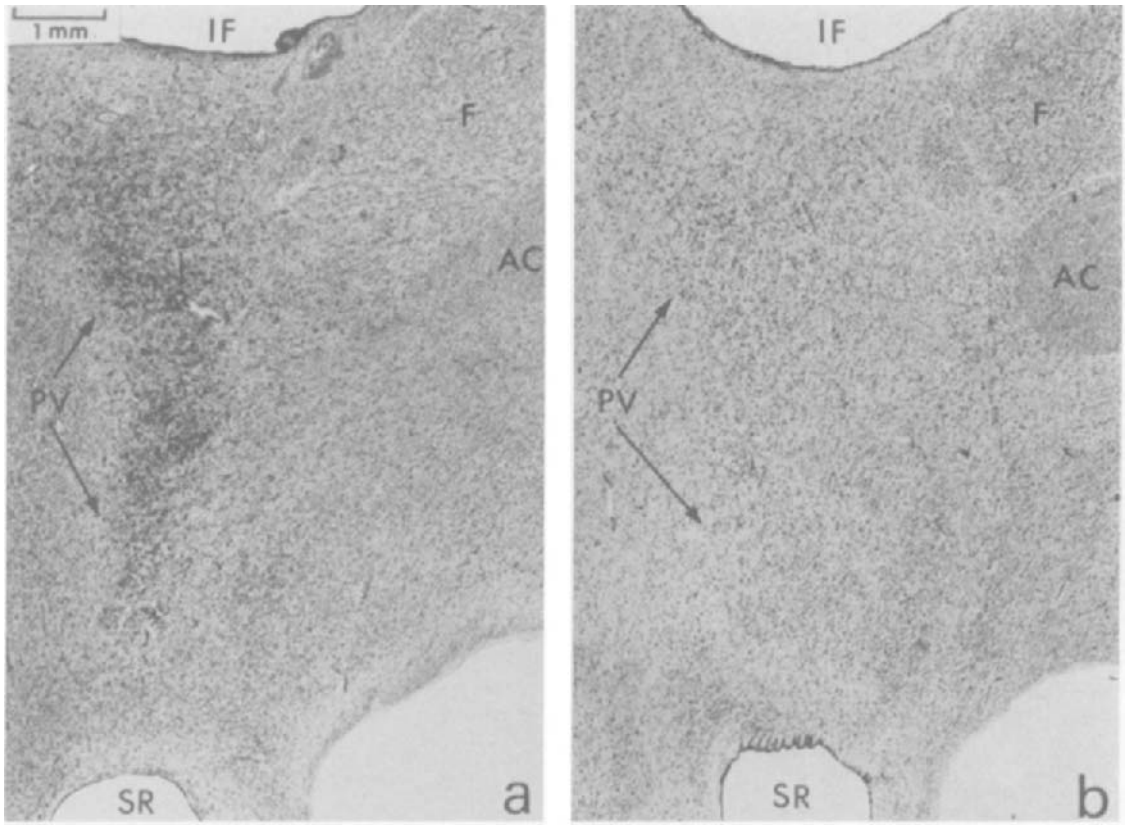

Fig. 17. Effects of hypophysectomy and of pituitary stalk section on the paraventricular nucleus. Parasagittal Nissl stained sections through the paraventricular nucleus of a normal subject (a), and of a patient who died 8 years after hypophysectomy (b). AC, anterior commissure; F, anterior column of fornix; IF, interventricular foramen; PV, paraventricular nucleus; SR, supraoptic recess of third ventricle.

ment of metastatic cancer of the breast or diabetic retinopathy. Many of the SON and PVN neurons are disintegrating and dying by the end of the second week after the operation and at 3 weeks a loss of nerve cells was definitely apparent. After 3 months, dying cells were seldom seen (Daniel and Prichard, 1972, 1975). Morton (1970) found a cell loss of 25% at 3 weeks after the operation, and after 3 months, 50% of the nerve cells had disappeared. In contrast to Daniel and Prichard (1975), Morton (1961, 1970) found a continuing cell loss until about a year after the operation, when approximately 20% of the nerve cells remained. Some discrepancies in literature concerning cell loss may be due to the fact that individual variability in cell number in the SON and PVN is considerable (see before). No close relationship has been found between the length of the remaining stalk and the number of residual cells in the SON and PVN. In the SON and PVN of stalk sectioned patients gliosis has been reported. An accumulation of Chomorri positive neurosecretory material within a week after operation was reported; this increased staining disappeared after a month (Daniel and Prichard, 1972, 1975).

Following suprasellar removal of the pituitary or stalk sectioning, a polyuria starts usually one day after the operation due to the acute damage of the hypothalamo-neurohypophysial system (Seckl et al., 1990). This phase was followed by a period of normal urine levels that could be explained by the release of preformed vasopressin. The occurrence

of permanent polyuria was frequent but unpredictable. After a month, the stalk has regenerated and has a nearly normal appearance. Patchy innervation and haemosideration granules are observed. After a year, the stalk is reinnervated throughout, although less abundantly and with a finer calibre fiber than can be found in a normal stalk. From animal experiments it is estimated that if less than 5–15% of the SON cells remained in the SON, polyuria would occur (Lipsett et al., 1956). In some cases where the pituitary stalk has been transected a newly formed small ectopic 'miniature neurohypophysis' is found at the proximal stump of the transected stalk. The ectopic posterior lobe secretes vasopressin and shows a high intensity signal on T1-weighed MRI images (Fujisawa et al., 1987a; Daniel and Prichard, 1975).

The immediate effect of stalk sectioning is a venous infarction of the neural tissue of the stump that is due to thrombosis of the long portal vessels. Because the arterial blood flow is not cut off by the operation, an intense congestion of the blood vessels in the stump occurs, as well as an extravasation of blood. Necrosis of neuronal tissue may involve irregular areas higher up, up to the junction of the stalk and the optic chiasm. The cells of the pars tuberalis usually survive. Haemorrhages may extend into the hypothalamus, around the infundibular recess (Daniel and Prichard, 1972, 1975).

Depression

Currently at least four hypothalamic peptidergic systems are considered to be involved in symptoms of depression, but the decreased activity of the suprachiasmatic nucleus and the alterations in the thyroid regulation will not be discussed here.

Corticotrophin-releasing hormone (CRH) and vasopressin-expressing neurons in depression

An increasing proportion of CRH neurons express also vasopressin as a sign of activation during the course of aging (Fig. 18). Major depressed patients and patients with a bipolar depression showed a much stronger CRH neuron activation than controls or Alzheimer's disease patients as appears from the increased total number

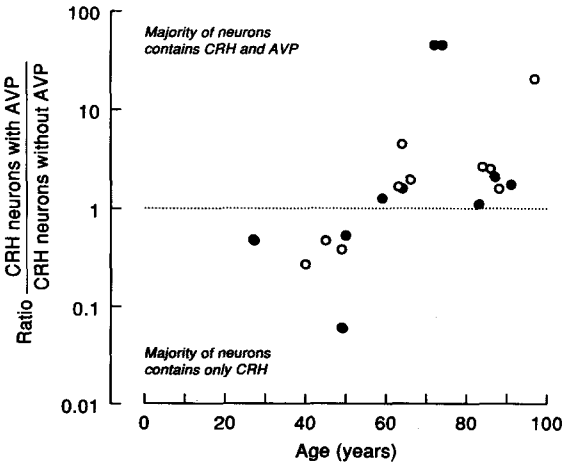

Fig. 18. Age-related increase in the ratios of the number of CRH neurons showing colocalization with AVP over those of CRH neurons not showing colocalization with AVP. Control subjects (●), Alzheimer patients (○). Correlations between age and ratio are highly significant (P=0.02) in controls and Alzheimer's disease patients and show Spearman's correlation coefficients of 0.71 and 0.72, respectively. Note that the majority of CRH neurons colocalize AVP above the age of approximately 60 years (from Raadsheer et al., 1994b, with permission).

of cells expressing CRH, the increased total number of CRH neurons showing vasopressin colocalization and the increased amount of CRH-mRNA in the PVN (Raadsheer et al., 1994c, 1995). The observation that the number of non-vasopressin-coexpressing CRH neurons increased more in major and bipolar depression than the number of vasopressin-coexpressing CRH neurons (Raadsheer et al., 1994c) seems to indicate that different subtypes of CRH neurons are present in humans and that these are activated differentially in depressed patients (Raadsheer et al., 1995). This view is supported by the finding of two subtypes of CRH neurons in the PVN of experimental animals (Whitnall et al., 1993). One type colocalized AVP and, in human, increases in activity with aging and projects to the median eminence, whereas the other type does not coproduce AVP and projects to the brain stem and spinal cord (Sawchenko and Swanson, 1982). However, this principle still has to be confirmed for the human

brain. Although in the rat these non-neuroendocrine neurons represent only a minor subpopulation of the CRH neurons in the PVN (Mezey et al., 1984; Swanson et al., 1983), our data indicate that, if the same principle goes for humans, this fraction may be considerably larger in our species.

At present there are various arguments suggesting that CRH might be a causal factor in the development of symptoms of depression. Firstly, there is a strong increase of CRH activity in major depression (Raadsheer et al., 1994c, 1995); the total number of CRH neurons of the major depressed patients was four times higher than in controls (Raadsheer et al., 1994c). In addition CRH-mRNA, as determined by quantitative in situ hybridization, was strongly activated in major depression (Raadsheer et al., 1995). Increased plasma cortisol levels and disturbed dexamethasone suppression, enhanced adrenal response to ACTH, blunted pituitary ACTH response to CRH and adrenal and pituitary enlargement in depression also indicate increased HPA-axis activity in this disorder (Krishnan et al., 1991a,b; O'Brien et al., 1996; Rubin et al., 1996). A second argument for the crucial role of CRH is that symptoms resembling depression, e.g. decreased food intake, decreased sexual activity, disturbed sleep and motor behavior and increased anxiety can be induced in experimental animals by intracerebroventricular injection of CRH (Holsboer et al., 1992). Thirdly, antidepressant drugs attenuate the synthesis of CRH (Fischer et al., 1990; Brady et al., 1991, 1992; Delbende et al., 1991; Reul et al., 1993) and the CRH concentrations in CSF in healthy volunteers (Veith et al., 1993) and the CRH levels in CSF of depressed patients (De Bellis et al., 1993). Lastly, a transgenic mouse model which has an overproduction of CRH appeared to have increased anxiogenic behavior, i.e. symptoms that are usually related with major depression that could be counteracted by injection of CRH antagonist (Stenzel-Poore et al., 1994).

The sum of these arguments leads to the CRH-hypothesis of depression, i.e. that the hyperactivity of a subgroup of CRH neurons that sometimes colocalizes AVP and does not project to the median eminence but into the brain may be activated in depression and cause symptoms in this disorder.

Oxytocin and vasopressin neurons

CRH and vasopressin are colocalized in an increased number of PVN neurons in depressed patients (Raadsheer et al., 1994c). We not only found an increased number of vasopressin coexpressing CRH neurons in depression, but also an increase in the total number of vasopressin and oxytocin expressing neurons in the PVN, of 56% and 23% respectively, indicating an increased production and a release of these peptides (Purba et al., 1996). Our observations thus confirm the postulation of Von Bardeleben and Holsboer (1989) that the action of CRH in depression is enhanced by vasopressin. Recently Van London et al. (1997) indeed reported increased levels of vasopressin in depressed patients. The melancholic patients had higher plasma levels than the non-melancholic patients. There appeared to be a slight relationship between plasma vasopressin levels and pychomotor retardation and a significantly inverse relationship between these levels and neuroticism. The oxytocin plasma levels of depressed patients showed only a trend towards higher levels in this study.

It should be noted that not only vasopressin, but also oxytocin may potentiate the effects of CRH (Yates and Maran, 1974; Gillies and Lowry, 1979; Carlson et al., 1982; Gillies et al., 1982; Vale et al., 1983; Muir and Pfister, 1988; Makara, 1992). In the rat, oxytocin neurons that coexpress CRH are present in the PVN (Sawchenko et al., 1984; Pretel and Piekut, 1990), but in the human PVN this coexistence has not been studied. Such a coexistence may be of particular importance, since animal experiments have implicated oxytocin as a possible central mediator of CRH-induced anorexias (Olson et al., 1991a,b). In addition to their endocrine actions, CRH and vasopressin may also act synergistically on behavior. In this way, increased vasopressin and oxytocin cell activity might contribute to mood changes, decreased food intake, and cognitive impairment (Legros and Ansseau, 1992; Swaab et al., 1995). A recent study showed that patients displaying clearly increased activity of the HPA-axis in mid-afternoon had elevated vasopressin levels, as did patients who had attempted suicide. These data therefore support the possible

synergistic effects of vasopressin and CRH, both at the level of the pituitary and of behavior (Inder et al., 1997). The activation of oxytocin neurons might also contribute to the decreased food intake observed in depressed patients, since the oxytocin cells of the PVN are putative satiety neurons of the brain (Swaab et al., 1995).

The idea that activation of the CRH and vasopressin neurons is related to the symptoms of depression, is reinforced by the observation of De Bellis et al. (1993), who found a significant decrease, not only of CRH, but also of vasopressin levels, accompanied by a decrease in the Hamilton Depression Scale ratings following fluoxetine treatment. The therapeutic effects of this serotonin reuptake inhibitor may thus be related to diminution of these two arousal-promoting neuropeptides.

Schizophrenia

Schizophrenia is considered to be a developmental disturbance due to genetic, viral or metabolic factors during pregnancy, or to obstetric complications (Geddes and Lawrie, 1995; Squires et al., 1995; Susser and Lin, 1992; Susser et al., 1996). The large number of structural abnormalities reported in schizophrenia agrees with the developmental hypothesis. Various changes in vasopressin and oxytocin have been reported in schizophrenia. Mai et al. (1993) observed strongly decreased numbers of neurophysin containing neurons in the PVN of untreated schizophrenic patients, but not in their SON. Unfortunately, these authors could, however, not distinguish between vasopressin- or oxytocin-neurophysin. Indeed, changes of water metabolism with possible involvement of vasopressin have been reported in schizophrenia. Compulsive water drinking is frequently found in chronic schizophrenics (Legros and Ansseau, 1992). Water intoxication may be a serious problem in many patients suffering from a chronic psychiatric illness, as are polydipsia and hyponatremia (Goldman et al., 1988; Verghese et al., 1993). These phenomena seem to be due to unexplained defects in urinary dilution, osmoregulation or water intake and the secretion of vasopressin. Upregulation of vasopressin receptors in the kidney has been presumed (Goldman et al., 1988). For reasons that are not quite clear, 3–5% of the chronic schizophrenic patients are hyponatremic and experience life-threatening episodes of water intoxication that may lead to seizures, delirium, irreversible neurologic defects, and death. Psychotic exacerbations are associated in this group of polydipsic schizophrenic patients with chronic hyponatremia with enhanced plasma levels of vasopressin. The exacerbated psychosis seemed to be responsible for the enhanced vasopressin release. In a group of closely matched normonatremic schizophrenic patients vasopressin levels rose a little, if at all (Goldman et al., 1997). In one patient with schizophrenia and inappropriate secretion of antidiuretic hormone, the hyponatremia ended after recovery from psychosis by electroconvulsive therapy (Suzuki et al., 1992). However, Frederiksen et al. (1991) did not observe a difference in hypothalamic vasopressin content in schizophrenic patients. There is, moreover, no consensus as far as circulating levels of neurohypophysial hormones in schizophrenic patients are concerned (Legros and Ansseau, 1992). On the one hand, reduced vasopressin levels and their corresponding neurophysin levels have been observed in CSF and plasma (Sarai and Matsunaga, 1989). By contrast, schizophrenic patients have been reported to excrete less water after water loading, which suggests vasopressin hypersecretion (Goldman et al., 1988; Legros and Ansseau, 1992). Plasma vasopressin levels were reported to be increased in acutely psychotic patients (Raskind et al., 1978). On the other hand, Beckmann et al. (1985) did not find differences in vasopressin levels in cerebrospinal fluid in schizophrenia. Increased oxytocin levels in schizophrenic patients have been confirmed by Legros and Ansseau (1992). However, it should also be noted that antipsychotic medicines might affect the activity of the hypothalamo-neurohypophysial system. Oxytocin levels increased after three weeks of treatment with neuroleptic drugs (Legros and Ansseau, 1992). Psychotropic drugs such as thiothixe, amitriptylene, thioridazine and chlorpromazine might cause inappropriate antidiuretic hormone secretion. Irreversible neurological symptoms and even coma have been reported as a result of such effects (Ajlouni et al., 1974; Tildesley et al., 1983; Ananth and Lin, 1987). Also animal experiments point to a stimula-

tion of supraoptic and paraventricular nuclei in rats treated with neuroleptics (Ireland and Connell, 1990). In addition, electroconvulsive therapy (ECT) results in a rise of vasopressin levels both immediately after ECT and one week after the last ECT (Narang et al., 1973). One must thus try to distinguish disease- and treatment-related effects in the reported hypothalamic alterations in schizophrenic patients.

The SON and PVN in aging and Alzheimer's disease

It's a fortunate person whose brain
Is trained early, again and again,
And who continues to use it
To be sure not to lose it,
So the brain, in old age, may not wane
Rosenzweig and Bennett, 1996

Various observations have provided evidence in support of our hypothesis that activation of neurons interferes in a positive way with the process of aging and with Alzheimer's disease, and may thus prolong the life span of neurons or restore their function. This hypothesis is paraphrased as 'use it or lose it' (Swaab, 1991). The neurons of the SON and PVN have been instrumental in the formation of this hypothesis, since they form a population of extremely stable cells in normal aging and in Alzheimer's disease. The classical Alzheimer changes are generally absent in the SON and PVN (Saper and German, 1987; Ishii, 1966). Despite the use of several antibodies, neither cytoskeletal alterations nor β/A4 plaques are generally found in the neurons of the SON of Alzheimer patients (Standaert et al., 1991; Swaab et al., 1992). Only in 5–8% of the old subjects studied were Alzheimer changes observed in the PVN and SON respectively (Schultz et al., 1997). These observations are in accordance with the idea that there was a relationship between metabolic activity and protection against aging and Alzheimer's disease. Although in the PVN of Alzheimer patients some neuronal and dystrophic neurite staining can be observed with cytoskeletal antibodies (Swaab et al., 1992), no β/A4 plaques are present (Standaert et al., 1991) and the total cell number in the PVN remains unaltered (Goudsmit et al., 1990). SON and PVN neurons are not only metabolically highly active throughout life, but are extra activated in senescence as well, as can be judged from the increase in the size of the vasopressin-producing perikarya (Fliers et al., 1985), nucleoli (Hoogendijk et al., 1985) and Golgi apparatus (Lucassen et al., 1993 Lucassen et al., 1994) (Fig. 19), and from the enhanced plasma levels of vasopressin (Frolkis et al., 1982; Rondeau et al., 1982) and neurophysins (Legros et al., 1980). It should be noted, though, that there are also reports that do not confirm an increased basal vasopressin plasma level in aging (Helderman et al., 1978; Rowe et al., 1980). The number of vasopressin-expressing neurons in the PVN increases during the course of aging, a change that is also interpreted as hyperactivation, and remains stable in Alzheimer patients (Van der Woude et al., 1995; Fig. 20). The increased activity of the SON and PVN during aging is most probably also the basis of the age-related decline in the frequency of the posterior pituitary bright spot found by MRI, a frequency estimated to decline with a rate of approximately 1% per year (Brooks et al., 1989).

Although after 24 h of water deprivation there is a deficit in thirst in healthy elderly men, osmoreceptor sensitivity as estimated by the vasopressin response to hypertonic saline increases with age, which is in agreement with the increased neurosecretory activity we observed. However, there is a failure of elderly people to release vasopressin in response to orthostasis and a paradoxical response to ethanol (Helderman et al., 1978; Robertson and Rowe, 1980; Rowe et al., 1980; Kirkland et al., 1984; Phillips et al., 1984). Alzheimer patients are at risk of dehydration due to a loss of the protective 'thirst' response. Plasma vasopressin levels were not different from controls after overnight dehydration, but these levels can be considered as inappropriately low for the level of serum osmolality (Albert et al., 1994).

The observation that the plasma vasopressin response to osmotic stimulation by hypertonic saline infusion is intact in Alzheimer patients. Peskind et al. (1995) supports the idea that this system remains largely unaffected in this condition. This observation also questions the impor-

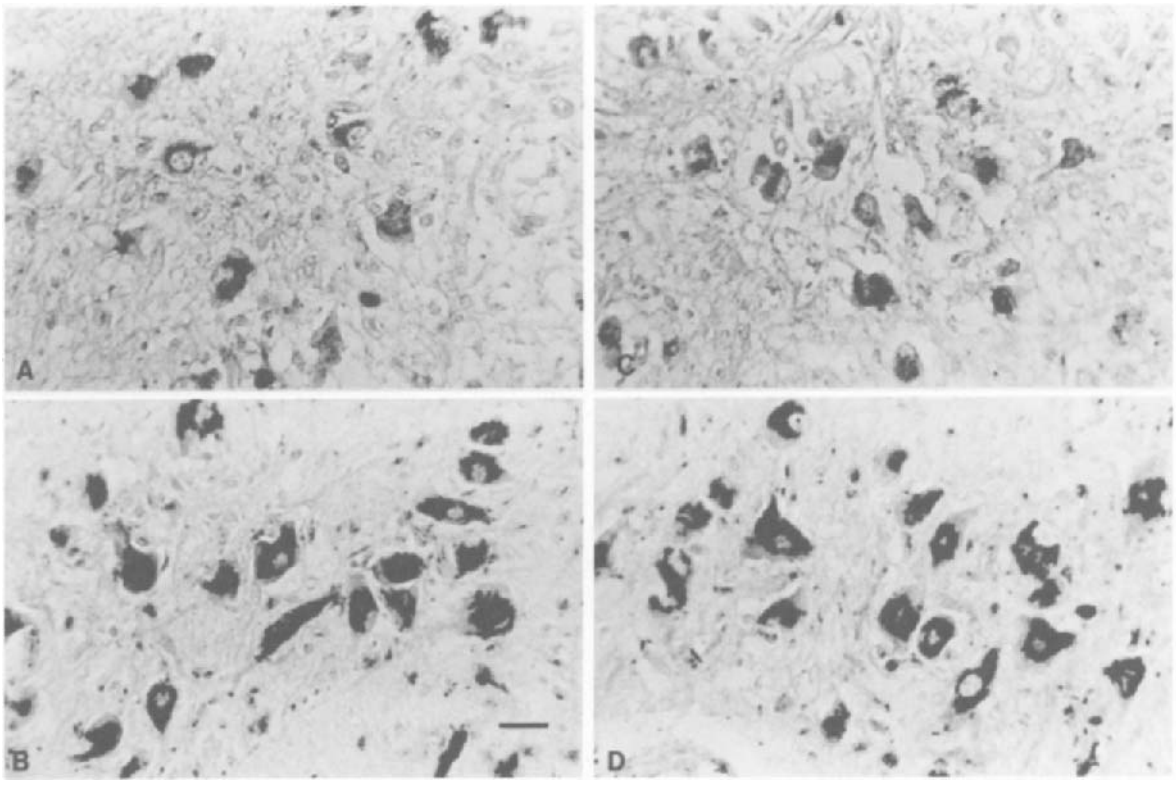

Fig. 19. Photomicrograph depicting Golgi apparatus staining in vasopressinergic SON neurons of the hypothalamus of (A) a 43-year-old control subject, (B) an 82-year-old control subject, (C) a 49-year-old Alzheimer patient and (D) an 81-year-old Alzheimer patient. Note the activation with age both in controls and Alzheimer patients as shown by the increased size of the Golgi apparatus. Scale bar, 28 μm (from Lucassen et al., 1994, with permission).

tance of the cholinergic system in the osmotic response of vasopressin neurons since this system was previously shown to be seriously affected in Alzheimer's disease. As appeared from animal experiments, the increased activity of SON and PVN neurons during the course of aging might be considered as a compensatory activation due to a loss of vasopressin receptors in the kidney (Fliers and Swaab, 1983; Ravid et al., 1987; Goudsmit et al., 1988; Herzberg et al., 1989). However, such a loss of kidney receptors for vasopressin still has to be proved in human aging. In normal elderly people thirst is significantly reduced. Water conservation and excretion are also impaired. However, the relationship between vasopressin and osmolality is unchanged, which suggests that the increased plasma osmolality seen in elderly people may be due to the kidney's reduced response to vasopressin (Ravid et al., 1987; Goudsmit et al., 1988). In this respect it is of interest that plasma osmolality may be a predictor of outcome in acutely ill elderly patients (O'Neill et al., 1990).

The number of oxytocin-expressing neurons in the PVN remains unaltered in aging and Alzheimer's disease (Wierda et al., 1991) (Fig. 21). In some projection areas, i.e. the hippocampus and temporal cortex of Alzheimer patients, the oxytocin concentrations even increased, whereas they remained unaltered in other areas (Mazurek et al., 1987), which is also in agreement with a stable oxytocin neuron population in aging.

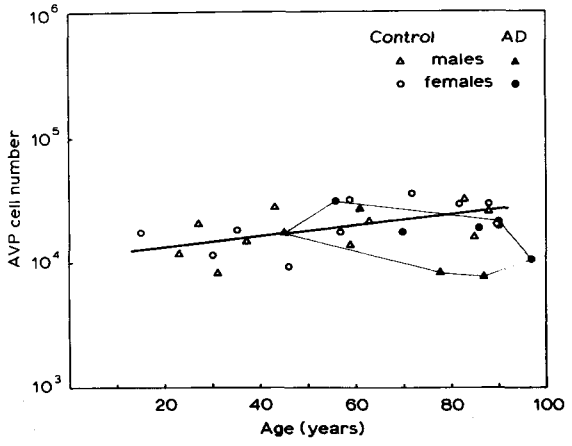

Fig. 20. Linear regression between vasopressin (AVP) cell number in the PVN and age. Data of male (Δ) and female (O) control subjects did not differ and were pooled. A significant correlation between age and cell number was found in control subjects ($r = 0.583$, $P < 0.01$; $n = 20$). Old control subjects had a significantly higher cell number compared with young controls. Values of male (▲) and female (●) Alzheimer's disease patients are delineated by a minimum convex polygon and were reduced compared to old controls. Note that the rise in AVP cell number with age in controls does not occur in Alzheimer patients (from Van der Woude et al., 1995, with permission).

Conclusions and summary

Vasopressin is produced in the human SON, PVN, SCN, diagonal band of Broca, bed nucleus of the stria terminalis, oxytocin is produced in the PVN and SON. Vasopressin and oxytocin are not only released as neurohormones in the neurohypophysis, to regulate kidney function and reproduction, in the median eminence together with CRH to release ACTH, but also as neurotransmitters or neuromodulators in other parts of the brain, influencing e.g. blood pressure, temperature regulation, cognitive functions, social attachment, paternal behavior, food intake and sexual behavior. Fetal vasopressin and oxytocin play a role in the adaptation to the stress of labor, and in speeding up the course of labor. In Prader-Willi syndrome the 50% drop in the number of oxytocin-expressing neurons is thought to be related to the insatiable hunger of these patients.

Developmental disorders affecting the vasopressin and oxytocin neurons include septo-optic dysplasia and anencephaly, while dystopia of the neurohypophysis rarely affects neurohypophysial function. Chronic alcohol consumption affects the vasopressin neurons mainly in the SON, but also in the PVN. Stalk sectioning causes a strong loss of neurons in both the SON and PVN. Pathology of the neurohypophysis includes acute haemorrhage and hypovolaemic shock, which may cause not only pituitary necrosis, but also a lesion of the SON and PVN, and granulomas, infections, granular cell tumors, metastatic carcinomas, hamartomas and other tumors. In addition, cytoskeletal Alzheimer alterations are sometimes found.

Diabetes insipidus might be of a primarily hypothalamic-familial nature due to mutations or, secondarily, to tumors, Langerhans cell histiocytosis, Wolfram syndrome, autoimmune processes, increased levels of cystine-aminopeptidase during pregnancy, septo-optic dysplasia, trauma, cerebrovascular lesions. It might also be caused by a molecular defect in the V_2-receptor, causing nephrogenic diabetes insipidus. Other drinking

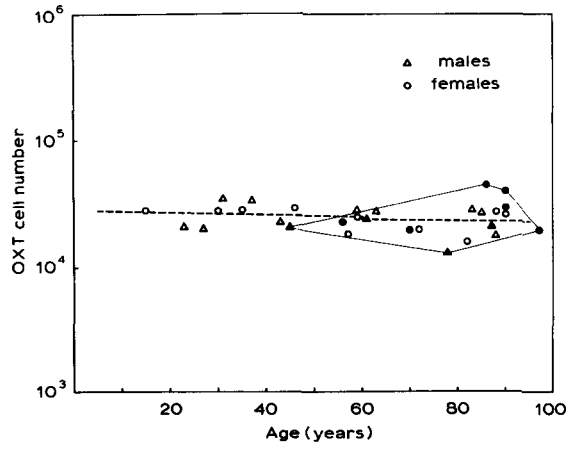

Fig. 21. Linear regression between oxytocin (OXT) cell number in the PVN and age. Data of male and female control patients did not differ and were pooled. No statistically significant correlations were observed in either young or old control subjects. Values of male and female Alzheimer disease patients (closed symbols) are delineated by a minimum convex polygon and were within the range of the controls (from Wierda et al., 1991, with permission).

disorders are primary polydipsia, adipsic or hypodipsic hypernatraemia, nocturnal diuresis, vasopressin hypersecretion in diabetes mellitus, inappropriate secretion of vasopressin (Schwartz-Bartter syndrome) and cerebral salt wasting.

In depression the increased number of CRH neurons that coexpress vasopressin may be related to mood disorder, the oxytocin cell activation to the eating disorder, the vasopressin increase to the risk of suicide and the decreased activity of the SCN to sleep disturbances. In schizophrenia changes in vasopressin and oxytocin have also been observed.

In aging, also in Alzheimer patients, the SON and PVN neurons are activated and remain generally intact. This supports the idea that activation of neurons interferes in a positive way in the process of aging and Alzheimer's disease, a concept that was paraphrased by us as 'use it or lose it'.

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I.J.A. Urban, J.P.H. Burbach and D. De Wied (Eds.)
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CHAPTER 6.2

Neuropsychological effects of vasopressin in healthy humans

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Animal research indicated that vasopressin (VP) exerts its principle behavioral influence, the improvement of memory formation, through an action on septo-hippocampal and connected limbic structures. Here human research is reviewed with the notion of a comparable effect of VP in healthy humans. Although the human studies yielded less consistent results than those in rats, they indicate that VP is able to improve declarative memory formation which is the type of memory essentially relying on hippocampal function. The effect appears to center on the encoding process for memory. In examinations of event-related brain potentials (ERPs) VP was consistently found to increase the 'mismatch negativity' (MMN) and the P3 components which are ERP potentials closely linked to the hippocampal processing of novel, unexpected and salient events. Enhanced processing of these stimulus aspects is considered to precipitate memory encoding. The regulation of voluntary selective attention and arousal do not appear to be primary targets of VP effects in humans. A mediation of effects by peripheral changes can be excluded since the central nervous effects were observed in studies using intranasal VP administration providing a direct access to brain functions.

Introduction

Extensive research in animals, mostly in rats, established a role of the neuropeptide vasopressin (VP) in learning and memory. The beneficial effects seen in rats stimulated numerous studies in humans designed to demonstrate a similar improvement of memory function following VP in this species. Overall these attempts showed less consistent results which at times appeared to be even discouraging. However, to judge the success of these research attempts in humans as completely negative is certainly premature and disregards the many pitfalls of human neuropharmacological studies which enhance the chance of an ambiguous outcome. This review will indicate that effects in humans of VP are, in fact, compatible with the view derived from animal research that the mediation of behavioral effects of VP relies primarily on hippocampal and related limbic structures. These structures play a significant role in the formation of memory also in humans.

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Neuroanatomy of the VP system

The neuroanatomy of the brain's VP system is described in detail in Chapter 1 of this book. The major hypothalamic sources of VP in humans, like in rats, are the nucleus supraopticus, the nucleus paraventricularis (both projecting to the neurohypophysis) and the nucleus suprachiasmaticus, known to function as oscillator circadian rhythmicity. The caudal paraventricular nucleus give rise to a descending projection distributing fibers mainly to the locus coeruleus, but also to other brain-stem structures including the dorsal raphe nucleus (involved in sleep regulation) and portions of the mesencephalic reticular formation involved in the regulation of arousal. Extrahypothalamic sources of VP have been identified in the bed nucleus of stria

terminalis, lateral septum, medial amygdala and in the locus coeruleus. These structures as well as the hippocampus also belong to the major targets of vasopressinergic fibers.

Brain receptors for VP appear to be primarily of the V_1 type (which contrasts with the fact that also V_2 agonists exert substantial central nervous effects). Distribution of VP receptors only partly matches with that of VP containing neurons (e.g. Phillips et al., 1990; Loup et al., 1991). Specific VP binding was found in numerous regions including the lateral septum, bed nucleus of the stria terminalis, the basal nucleus of the amygdala, the dentate gyrus of the hippocampus and several hypothalamic nuclei including the nucleus suprachiasmaticus and the arcuate nucleus. In the human thalamus, VP binding appears to be restricted to the midline nuclei and adjacent intralaminar nuclei (Loup et al., 1991).

Localization of VP effects in the rat brain

In animals, the significance of selective brain structures for the behavioral effects of VP has been proven using localized administration of the peptide, of selective receptor agonists and antagonists, and of antisera to the peptide as well as by local assessment of VP turnover. Based on these techniques, the septo-hippocampal system has been revealed to be the primary structure mediating effects of VP on memory. In rats, the effects of VP on memory have been established mainly by measuring passive avoidance behavior and resistance to extinction of active avoidance behaviors. For example, VP administered to the dorsal and ventral hippocampus, to the dentate gyrus, the dorsal septal nucleus as well as to the dorsal raphe nucleus improved retention of the passive avoidance response while VP antiserum injected immediately after the training session into some of these regions impaired retention of the avoidance response (for a review see Engelmann et al., 1996). Likewise, an increased resistance to extinction of active avoidance responses has been reported after intrahippocampal injections of VP agonists (Ibragimov, 1990). However, not only improving effects on learning and memory have been reported following local activation of VP receptors. For example, spatial learning in the Morris Water Maze has been found to be adversely affected by intraseptal VP administration (Engelmann et al., 1992).

Of course, observations on learning and memory of avoidance behaviors in rats can hardly be transferred directly to humans. Nevertheless, these results provide a useful frame of reference as to the question which neuroanatomical structure and which cognitive function could be most sensitive to the effects of VP. Results from animals clearly suggest that the septo-hippocampal system is the principle structure mediating the regulatory influence of VP on more complex behaviors. Although less essential, the amygdala may also contribute to some of the behavioral effects of the peptide. All of these structures have been also linked to defined aspects of human behavior. Thus, there is ample evidence from studies in brain lesioned humans that the hippocampal formation supports the formation of declarative memory (Squire, 1992). Moreover, the septo-hippocampal system is involved in the regulation of orienting towards novel and unexpected stimuli (Vinogradova, 1975; Tulving et al., 1994).

Investigating effects of VP in humans – Pitfalls and peculiarities

Undoubtedly, the relevance of investigating behavioral effects of VP in humans derives from the direct impact of these results for potential therapeutic use. In light of the specific functional and anatomical properties of the human brain and mind it must be cautioned against any a priori generalization of findings from rodents to humans. However, the experimental analysis of neuropeptide effects in humans faces a number of outraging limitations which are probably the cause for the clear preponderance of work done in animals. The restrictions of human research result primarily from the rather limited possibilities of gaining experimental access to the brain's own VP system. In contrast to animal studies where local manipulation of VP containing brain structures is the method of choice, human trials rely on systemic administration of the peptide. Yet, it is widely accepted that VP does not readily pass the blood—brain barrier (BBB; Ermisch et al., 1993), although evidence for

a saturable carrier mediated transport of the molecule across the BBB exists (e.g. Banks and Kastin, 1987; Zlokovic et al., 1990). Apart from a carrier-mediated transport, systemic VP may enter the brain locally via the circumventricular organs which lack a BBB and are densely vasculated. In some of these sites such as the area postrema and the median eminence VP receptors have been identified (Loup et al., 1991; Ermisch et al., 1993).

Although these routes could mediate central nervous effects of systemic VP, side effects of the hormone in the body periphery mask and interact with the actions on the brain, in particular at higher plasma concentrations (e.g. Ettenberg et al., 1983; Ebenezer, 1994). Therefore, it has been attempted to avoid peripheral side effects by the use of analogs and fragments of VP. For example, Deamino-D-arginine-VP (DDAVP) is a selective agonist of the V_2 receptor which lacks the vasoconstrictory effects of natural VP. Des-glycinamide-AVP (DGAVP) lacks both vascular and renal effects and appears to activate selectively central nervous VP receptors. A rather direct stimulation of the central nervous VP system can be achieved also by systemic injection of hypotonic saline solution resulting in increased endogenous VP release in the septum, hippocampus and nucleus supraopticus (e.g. Landgraf et al., 1988). However, this approach so far has not been adopted to assess behavioral effects of VP in humans.

Intranasal administration of VP

In human studies, a preference has been developed to administer VP intranasally. This route was considered to be least invasive in order to enhance systemic VP levels. Riekkinen et al. (1987) showed in patients undergoing diagnostic lumbar puncture that after intranasal administration DGAVP enters the cerebrospinal fluid (CSF). Although levels in plasma were on average more than ten-fold higher than in CSF, the amount of DGAVP that accumulated in CSF was considerable. Curiously, it was distinctly higher than would have been expected from animal and patient studies testing cerebrospinal uptake of VP after subcutaneous and intravenous injection of the substance (Mens et al., 1983; Stegner et al., 1983; Sorensen et al., 1984; Ermisch et al., 1985). Since the olfactory nerves are surrounded by a space that contains liquor and is continuous with the subarachnoid space, after intranasal administration the peptide may diffuse directly into the cerebrospinal compartment through the olfactory epithelium. This view is substantiated by findings in rats and primates providing evidence that larger tracer molecules, such as horseradish peroxidase, after intranasal administration, pass freely through intercellular junctions of the olfactory epithelia to reach extracellularly the fiber layer of the olfactory bulb within minutes (Balin et al., 1986). Peroxidase in those studies diffused out of the olfactory fiber layer over time, presumably as a consequence of bulk flow of CSF. Likewise, several viruses and drugs like cephalexin have been demonstrated to enter the brain via the olfactory nerve (e.g. Sakane et al., 1991; Barnett and Perlman, 1993). In humans, peak insulin concentrations in CSF sampled with a lumbar catheter, were reached within 20 min after intranasal administration of the peptide, in the absence of any concomitant increase in plasma insulin levels (Kern et al., 1998).

Convergent functional evidence that effects of VP after the intranasal administration on human cognitive function derive from a direct access of the peptide to the brain has been provided by Pietrowsky et al. (1996). That study in healthy men compared the effects of arginine-VP (VP) on event-related brain potential (ERP) responses to tones, after the intranasal and the intravenous route of administration. The dose and rate of the intravenous infusion were chosen so that increases in plasma concentrations of VP were equal to or substantially higher than those observed following the intranasal route of administration of 20 IU VP. While intranasal VP induced a distinct increase in amplitude of the P3 component of the ERP, intravenous infusion of VP at all plasma concentrations remained completely ineffective (Fig. 1). Comparable results were revealed by several other studies exploring the intranasal route of administration for other peptides, such as corticotropin-releasing hormone, cholecystokinin and insulin (Pietrowsky et al., 1997; Kern et al., 1998).

These studies support the view that after intranasal administration in humans, peptides have a facili-

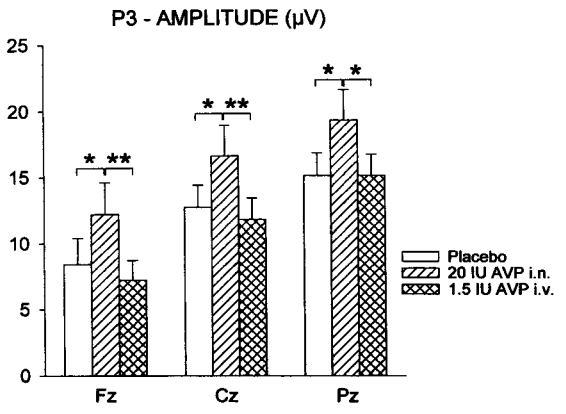

Fig. 1. Mean (± SEM) amplitude (μV) of P3 after target stimuli of an auditory oddball task. Recordings were obtained from electrodes attached over mid-frontal (Fz), mid-central (Cz), and mid-parietal (Pz) cortical areas following administration of placebo (empty columns), intranasal administration of 20 IU AVP (hatched columns), and intravenous administration of 1.5 IU VP (cross-hatched columns). $*P < 0.05$, $**P < 0.01$, for pairwise comparisons. Note, although plasma levels of VP after intravenous administration of the peptide exceeded those after intranasal administration, P3 was increased after the intranasal administration only. The intravenous route of administration remained ineffective also with lower doses of 0.025 and 0.1 IU AVP, which yielded increases in plasma VP concentration closely comparable with those after intranasal administration of 20 IU VP. (From Pietrowsky et al., 1996.)

tated access to the CSF compartment. Nevertheless, further investigations of concentrations of VP in liquor and plasma after intranasal VP administration are indicated. Also, when examining the mechanisms of intranasal VP administration, the possibility of a nose–brain transport within olfactory sensory neurons must still be considered, although this route appears to be much slower (Balin et al., 1986). Moreover, in the human olfactory bulb no specific binding sites for VP have been found (Loup et al., 1991). A problem of greater relevance in this context is the xenobiotic metabolism in the nasal cavity. It cannot be ruled out, so far, that prior to its uptake the VP molecule is broken down into centrally active fragments (such as VP 4–9) which mediate the neurobehavioral changes (Burbach et al., 1983; Dahl and Hadley, 1991).

In summary, the presently available data support the concept that after intranasal administration of VP the peptide gains direct access to brain functions, even though the exact mechanisms of this pathway remain to be clarified. This does not exclude that after intranasal administration a portion of VP passes to the vascular compartment. However, this portion is small, and effects on brain functions after intranasal VP are usually detectable at doses which do not exert any substantial effects on peripheral functions such as blood pressure, diuresis, and pituitary-adrenal secretory activity. Therefore, the nose-brain pathway could provide a unique chance to examine direct effects of VP on the brain and behavior in the absence of any contamination by hormone-induced changes in the body periphery.

Experiments with healthy humans

Even if exogenous VP gains access to the brain, its interaction with the endogenous central nervous VP system has to be considered. Experimental tasks may induce pronounced activation of this system thus prohibiting the emergence of effects caused by the exogenous VP (for related work in animals, see Engelmann et al., 1994). This consideration appears to be particularly relevant for the examination of effects of VP in healthy humans. A young student, the typical subject in such experiments will be highly motivated upon instruction to learn a list of words. In turn, brain regulation will be optimized to cope with the task, possibly also including a strong activation of endogenous VP release. A 'ceiling effect' would thus prevent the emergence of any effect of exogenous VP administration. Hence, there may be a better chance to reveal effects of VP under suboptimal conditions as present, for example, in healthy elderly subjects (Swaab, 1995), or with less demanding task situations which do not lead to exhaustive activation of the VP receptor system. This view is supported by observations of stronger effects of VP in subjects showing poor performance on a certain task than in high performers (e.g. Till and Beckwith, 1985).

Memory

A concept

Memory refers to more or less stable internal representations of stimuli and events. Commonly, immediate memory, short-term memory (STM), and long-term memory (LTM) are distinguished with reference to the time span an internal representation is stored in these systems (Fig. 2). Immediate memory (some seconds) refers to the short phase of acute sensory stimulus processing and the associated excitation within afferent and cortical sensory neuron assemblies. Conscious processing requires that the stimulus representation gains access to STM (covering minutes). Since STM has a limited capacity, some kind of selection takes place before a stimulus enters STM. Within STM, the representation of an event can be maintained by rehearsal. For example, a telephone number can be kept in mind just by constantly repeating the sequence of numbers. However, usually in STM stimuli are not just kept per se, but some kind of operation is performed on them, i.e. they are linked to other stimuli and to a certain response. For this reason, the term 'working memory' is preferred with reference to the conscious processing of stimuli within STM. Memory encoding and consolidation refers to the transfer of information from STM to LTM for permanent storage, resulting in persistent memory traces representing events and experiences. Consolidation depends on the time information is kept in STM. Thus, rehearsal of a telephone number in STM may gradually engrave the respective memory traces. Memory encoding depends on the level of stimulus processing. For example, to decide whether a word fits in a sentence requires semantic analysis and, hence, a much deeper processing than to decide whether or not this word is written in capital letters. Consequently, LTM of words after semantic processing is superior to that after mere structural processing of the letter features (Craik and Lockhart, 1972). Deeper processing means that a greater number of associative connections to related representations (e.g. to those of the other words of the sentence) are established facilitating integration of the event into the network of representations in LTM (Morton, 1969; Kroll and Klimesch, 1992). Ongoing processes of memory encoding and consolidation consume part of the limited processing capacities of LTM, and can thus interfere with concurrent operations in STM (Walker, 1958; Walker and Tarte, 1963).

The retrieval process is often viewed as a spreading of activation into certain ranges of the network

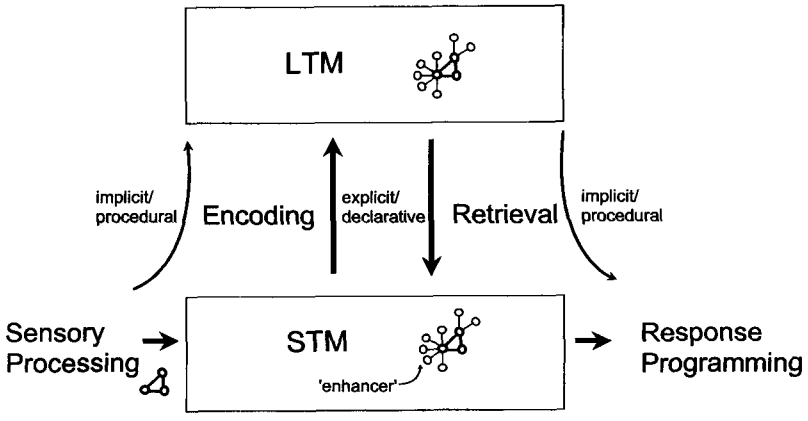

Fig. 2. Psychological concept of memory processes. STM, short-term memory or working memory; LTM, long-term memory. The hippocampus is supposed to enhance predominantly activated representations in STM (as indicated by the thick trace in the representational network) thereby supporting encoding of respective events in LTM. Refer to text for further explanations.

of representations in LTM (Anderson, 1983). Retrieval, i.e. the access to a memory trace depends on its strength and its connectivity with associated representations. Retrieval can be assessed by recognition or recall performance. Recognition requires the subject to judge if a stimulus presented is novel or familiar. Recall on the other hand requires active search within LTM which is facilitated by an increased number of associative connections to the target representation. Thus, unlike recognition reflecting the strength of a representational memory trace, recall performance additionally reflects its accessibility via associative links.

Apart from memory formation mediated via STM, which occurs when learning is explicitly required, memory formation as well as retrieval can occur also implicitly without major contributions of STM function. A distinction sharing many similarities with that between explicit and implicit memory processes is that between, respectively, declarative and procedural memories. The declarative memory system underlies the acquisition and retrieval of new facts (semantic memory) and specific events (episodic memory). Procedural memory refers to the acquisition of perceptual and motor skills by repeated practicing of the task (e.g. learning how to ski). In contrast to procedural and implicit memory, declarative memory essentially relies on hippocampal and adjacent entorhinal, parahippocampal and perirhinal structures (Squire, 1992). The hippocampus in this context probably acts to enhance the signal-to-noise ratio in STM thereby facilitating consolidation of predominantly activated representations. Predominant activation may derive from the biological significance of a stimulus, its novelty, rare occurrence and, in experimental situation, from its relevance for task performance.

VP and memory

Considering the various memory processes and their interaction with other functions like arousal and attention, not mentioned so far, one cannot expect that a peptide like VP ubiquitously improves memory. Correspondingly, in light of the many different task paradigms used to explore VP effects on the various aspects of memory function, one cannot expect far reaching consistencies across results from different experiments. It is the more surprising that the majority of studies in healthy humans considered here report on improving effects on aspects of memory formation following administration of VP, and with a few exceptions there is no hint for an impairing effect of VP. A summary of the studies assessing effects of VP on STM and LTM function, is given in Table 1.

Declarative versus procedural memory

Assuming an action of VP via hippocampal function declarative memory is expected to be more sensitive to the peptide's influence than procedural memory. Unfortunately, in most studies only declarative memory was tested, and there are only few studies of non-declarative memory which all failed to indicate an influence of VP. One of these studies was conducted by Medvedev et al. (1981) who examined the effects of a single intranasal administration of VP (25–35 μg) on two tests of declarative memory in healthy men. One required the immediate and delayed (after 1 h) recall of a list of pseudowords (consonant–vowel–consonant trigrams). On the other task (Benton Test), subjects had to draw geometrical figures which they had just memorized. In addition, a task of implicit learning was employed which required the unexpected recall of individual numbers previously visualized while performing mental arithmetics. VP improved delayed recall of pseudowords and the reproduction of the geometrical figures. In contrast, implicit learning performance was not affected.

Complementing evidence provides a comparison of studies by Fehm-Wolfsdorf et al. (1983, 1984b, 1985). In all of these studies effects of lysine-VP (LVP) on recall of word lists was tested. In the 1983 and 1984 studies, LVP administered intranasally at doses of, respectively, 10 IU for 4 days and 20 IU for 3 days, enhanced immediate recall (although due to thorough practicing of the task in the latter study improvements of the placebo group somewhat obscured the effect of LVP). In the third study (Fehm-Wolfsdorf et al., 1985), the paradigm was changed to an implicit learning task: upon presentation, every word was to be rated on a

five-point scale with respect to certain lip-movements associated with speaking the word. No explicit learning instruction was given, but retrieval of the words from memory was explicitly required. LVP (20 IU, intranasally for 3 days) had virtually no effect on recall in this task. However, definite conclusions can not be drawn from this finding, since recall in this study also remained unaffected after an emotional evaluation of the word meaning.

In a recent study, Pietrowsky et al. (1998) examined effects of intranasal administration of 40 IU VP on delayed recall of paired word associates. Recall on this task which is a classical task of declarative memory, is cued by presenting the first word of each pair. Effects were compared with those on the acquisition of mirror tracing skills which is a classical task of procedural memory and requires the subject to trace as fast and as accurately as possible a figure, with visual feedback enabled by a mirror only. VP distinctly improved recall of words while retrieval of mirror tracing skills remained unaffected.

During implicit and procedural learning those stimulus features that are to be learned and retrieved later, will only rudimentarily enter STM and respective processes of encoding. Moreover, neuroimaging studies in healthy humans and studies in patients with temporal lobe lesions have indicated that memory encoding involves activation of the hippocampus and surrounding temporal lobe structures only during explicit learning (e.g. Squire, 1992; Tulving et al., 1994; Mayes et al., 1997). Thus, in contrast to the explicit, declarative learning procedures, implicit learning with respect to the target stimulus characteristics appears to invoke a non-declarative type of memory processing which is less supported by hippocampal activity. With this background, the studies indicate, under comparable treatment conditions, improved declarative memory after VP in the absence of any effects on indicators of implicit and procedural memory, in fact, they support a primary action of the peptide on memory function of the hippocampus. Nevertheless, it should be mentioned also, that some studies failed to reveal effects of VP in classical tasks of hippocampal memory (e.g. in tasks of spatial memory, Posmurova et al., 1983).

Retrieval from long-term storage

To discriminate effects on retrieval from those on memory encoding and consolidation, effects of VP given before retrieval have to be compared with the effect obtained following administration at the time of acquisition. This approach has been adopted in only a few studies which overall failed to demonstrate a sensitivity of retrieval to the influence of VP. Till and Beckwith (1985) investigated the effects of DDAVP (60 μg, intranasally) on immediate and delayed recall of sentences in a conventional cross-over design, i.e. one group of subjects received DDAVP before the first session and placebo on the second, while for the other group this order was reversed. In the case of a separate influence on retrieval DDAVP given prior to the second session should improve recall of material learned during the first session. This effect was not revealed. DDAVP only improved recall (in subjects with low verbal abilities) when given prior to learning. Likewise, in experiments of Fehm-Wolfsdorf et al. (1984b) LVP administered prior to retrieval failed to facilitate recall of materials learned 1 week before.

A different test of retrieval from LTM was used by Nebes et al. (1984). In this tests, the subject on each trial was presented with a category name (e.g. 'fruit') followed by a letter (e.g. 'p') which had to be answered as fast as possible by naming aloud an example of the category (e.g. 'peach'). Retrieval in this test requires active search in LTM while encoding is not demanded. A 1-week intranasal treatment with DDAVP did not affect performance on the task. While other studies also failed to find effects of VP in comparable tests (e.g. Millar et al., 1987), there were a few reports of a beneficial effect of VP on tasks such as finding synonyms for presented words (Medvedev et al., 1981; Weingartner et al., 1981a). Yet, overall results do not suggest that VP exerts a strong and specific effect on the retrieval process.

Memory encoding and consolidation

Numerous observations suggest that the effect of VP on memory encoding and consolidation is more potent than on retrieval. A good example is

Table 1
Summary of studies on memory and attention[a]

Study	N/sex/	Daily dose design	Duration (days)	Tota ldose	Effects
Medvedev et al. (l981)	20/m within	25–35 μg VP i.n.	1 day	25–35 μg	Immediate recall (pseudowords) Ø Delayed recalls ↑ Reproduction of visual patterns ↑ Implicit memory Ø Word fluency ↑
Weingartner et al. (1981b)	8/m, 4/f group	30–60 μg DDAVP i.n.	14	1260–2520 μg	Free recall (words) Cued recall ↑
Beckwith et al. (1982)	45/m group	60 μg DDAVP i.n.	1	60 μg	Benton test Ø Concept formation ↑
Jenkins et al. (1982)	10/f, 4/m group	160 μg DGAVP i.n.	14	2240 μg	Immediate recall (words) Ø Delayed recall Ø Word recognition Ø Digit-span Ø
Laczi et al. (1982)	2/m, 8/f within	10 IU LVP i.m.	1	10 IU	Paired-associate-learning ↑
		41 μg DDAVP i.m.	1	4 μg	Immediate recall (names, numbers) ↑ Spatial memory (maze-test) ↑
		20 μg DDAVP i.n.	7	140 μg	Bourdon test ↑
Beckwith et al. (1983)	15/m within	60 μg DDAVP i.n.	1	60 μg	Sternberg task ↑
Fehm-Wolfsdorf et al. (1983)	20/m group	10 IU LVP i.n.	4	40 IU	Immediate recall (words) ↑ Delayed recall Ø
Laczi et al. (1983)	2/m, 7/f within	3 μg DGAVP i.m.	1	3 μg	Paired-associate-learning
		30 μg DGAVP i.m.	1	30 μg	Immediate recall (names, numbers) ↑
		80 μg DGAVP i.n.	7	560 μg	Spatial memory (maze-test) ↑ Bourdon test ↑
Posmurova et al. (1983)	18/m, 18/f within	8 μg DDAVP i.m.	1	8 μg	Recall of stories ↑
		100 μg TGLVP i.m.		100 μg	Spatial memory Ø Tactile memory ↑
Beckwith et al. (1984)	64/m,64/f group	60 μg DDAVP i.n.	1	60 μg	Recall of sentences ↑
Fehm-Wolfsdorf et al. (1984a)	1/f within	40 IU LVP i.n.	6	240 IU	Free recall (words) Ø Sternberg task Ø Stroop task ↑
Fehm-Wolfsdorf et al. (1984b)	30/m group	20 IU LVP i.n.	3	60 IU	Immediate recall (words) (↑) Delayed recall (↑)
Nebes et al. (1984)	48/m within	1030 μg DDAVP i.n.	8	210 μg	Cued recall (words) ↑ Recognition Ø Sternberg task ↑

Fehm-Wolfsdorf et al. (1985)	10/m, 24/f group	20 IU LVP i.n.	3	60 IU	Recall after implicit learning Ø Immediate recall (words) Ø
Till and Beckwith (1985)	42/m within	60 μg DDAVP i.n.	1	60 μg	Recall of sentences ↑
Guard et al. (1986)	20/m, 20/f group	23 IU LVP i.n.	15	345 IU	Immeditate recall (words) Ø
		30 μg DDAVP i.n.	15	450 μg	Visual recognition Ø Reproduction of visual patterns Ø Digit-span Ø
Sahgal et al. (1986)	24/m group	20, 60 μg DDAVP i.n.	1	20, 60 μg	Cross-out concentration test Ø
Beckwith et al. (1987)	40/m group	60 μg DDAVP i.n.	1	60 μg	Recall of prose ↑
Millar et al. (1987)	36/m group	40 μg DDAVP i.n.	1	40 μg	Free recall (words) ↑ Element-category classifications Ø
Snel et al. (1987)	20/m group	0.1–10 μg DDAVP i.n.	5	14.4 μg	Free recall (words) Ø Visual recognition Ø Concentration (↑)
Pietrowsky et al. (1988)	13/m within	6 mg DGAVP i.n.	1	6 mg	Immediate recall (words) ↑
Beckwith et al. (1990a)	40/m, 40/f group	60 μg DDAVP i.n.	1	60 μg	Immediate recall (words) (↑) Stroop test ↓
Beckwith et al. (1990b)	70/m group	5, 15, 30, 60 μg DDAVP i.n.	1	5, 15, 30, 60 μg	Recall of sentences ↑ (60 μg) ↓ (15 μg)
Bruins et al. (1990)	6/m, 6/f group	2 mg DGAVP i.n.	1	2 mg	Immediate recall (words) ↑ Visual recognition Ø
Bruins (1991) (paper 4)	57/f, m group	1 mg DGAVP i.n.	20	20 mg	Immediate recall (words) Ø Delayed recall Ø Delayed recognition ↑ Face recognition ↑ Sternberg task Ø
Bruins (1991) (paper 5)	36/m group	1 mg DGAVP i.n.	20	20 mg	Immediate recall (words) ↑ Delayed recall ↑ Delayed recognition Ø Recall of prose Ø Sternberg task Ø
Bruins et al. (1992)	20/f group	2 mg DGAVP i.n.	1	2 mg	Immediate recall (words) Ø Delayed recognition ↑ Visual recognition Ø
Beckwith et al. (1995)	45/m group	60 μg DDAVP i.n. (after acquisition)	1	60 μg	Recall of prose Ø
Bruins et al. (1995)	20/m, 27/f group	2 mg DGAVP i.n.	1	15 mg	Immediate recall (words) ↑
		1 mg DGAVP i.n.	13		Delayed recall Ø Delayed recognition ↑ Visual recognition Ø Sternberg-task ↑

Table 1 (*continued*)

Study	N/sex/	Daily dose design	Duration (days)	Total dose	Effects
Perras et al. (1997)	15/f, 11/m group	40 IU VP i.n.	84	3360 IU	Initial recall (words) Ø Recall after interference ↑
Pietrowsky et al. (1998)	20/m within	40 IU VP i.n.	1	40 IU	Delayed recall (paired words) ↑ Mirror tracing Ø

[a] Sex: m, male; f, female. Design: within, subject comparison; group, design; for VP and LVP 1 IU is about 2.5 μg; i.n., intranasally; i.v., intravenously; i.m., intramuscularly; ↑, improved; ↓, impaired; Ø, no effect. Tasks of the respective studies are not comprehensivelv listed.

provided by the experiments of Bruins et al. (1990). To explore effects of DGAVP (2 mg, intranasally) on memory encoding, she adopted the 'restrictive reminding method' (Buschke, 1974; Buschke and Altman-Fuld, 1974). The task consisted of the presentation of 15 tape-recorded words which were to be recalled immediately. In the subsequent nine trials, the experimenter presented only those words that were not recalled previously. The major aspects of memory encoding discriminated with this test are as follows: (i) 'initial storage' as determined by the items recalled on the first trial, (ii) the 'rate of storage' as determined by the number of trials and presentations needed until each word is recalled at least once, (iii) 'short-term storage', i.e. those items recalled only once, and (iv) 'long-term storage' with consistent retrieval, i.e. repeated retrieval without failures on either the first (initial list learning) or any subsequent trial. DGAVP facilitated initial storage, rate of storage, short-term storage, and also on long-term storage of the initial list learning. Comparable results were obtained in a second study after subchronic administration of DGAVP (1 mg per day over 20 days; Bruins et al., 1995). In that study, the effects were limited to men and were not revealed in women which may be related to a generally lower verbal performance in men than women. Bruins et al. (1990) took the pattern of effects to suggest that DGAVP facilitates the process of storage in particular, i.e. memory encoding.

Related results were revealed by Fehm-Wolfsdorf et al. (1983) in a word list learning paradigm. In this study, LVP enhanced recall of the initial few words in a list of 15 words (primacy effect), but tended to diminish recall of the last items (recency effect). The primacy effect is commonly considered a reflection of recall from LTM whereas the recency effect reflects direct recall from STM. Accordingly, improved recall of words already stored in LTM can be viewed as confirmatory to an enhancing effect of LVP on processes of memory encoding. Subsequent studies confirmed an improvement of recall of word lists after VP, although the pattern of the Fehm-Wolfsdorf study was not exactly replicated. Thus, Millar et al. (1987) found a generally improved recall after DDVP with, however, no indication for a dependency of the effect on the serial position of the word. Pietrowsky et al. (1988) obtained a completely opposite pattern with, an attenuated primacy effect but an improved recency effect after DGAVP administration.

Contrasting with these findings, quite a number of experiments failed to reveal any effect of VP on immediate recall in list learning tasks (Jenkins et al., 1982; Fehm-Wolfsdorf et al., 1985; Guard et al., 1986; Snel et al., 1987). However, this inconsistency is not surprising if it is taken into account that the process of memory encoding consumes part of the limited processing capacities of STM (Walker, 1958; Walker and Tarte, 1963). In turn, arousing energy is lacking for retrieval processes when consolidation processes are still going on, which is the case when immediate recall is demanded.

Delayed retrieval

Assuming that immediate recall can interfere with ongoing consolidation, more distinct effects of VP could be expected with delayed recall. Thus, Medvedev et al. (1981) observed improved recall of pseudowords following VP only with delayed but not immediate recall. Bruins et al. (1992) measured recall and recognition of words 75 min, and 1 week after learning in women. DGAVP (2 mg) administered intranasally prior to acquisition enhanced recognition 1 week later, but not 75 min after the learning phase. A superior effect of acute treatment with DGAVP on delayed recognition, as compared with measures of immediate retrieval, was confirmed in a further study of this group (Bruins et al., 1995), although the effect remained restricted to the male participants. Subchronic administration of DGAVP, also tested in this study, facilitated both initial recall as well as delayed recognition. Of course, also negative outcomes have been reported for delayed retrieval conditions (e.g. Jenkins et al., 1982; Fehm-Wolfsdorf et al., 1988). However, in those studies measures of immediate recall also remained unaffected (or were not at all assessed) suggesting a general absence of memory effects.

These results converge to the conclusion that beneficial effects of VP on retrieval are more

consistent with delayed than immediate retrieval, which could be due to a fading interference with memory encoding processes continuing some time after the acquisition. On this background, it could be expected that the peptide improves memory also when administered shortly after the acquisition phase when processes of consolidation persist without interference from acute learning. However, Beckwith's group could not demonstrate any influence of a post-learning administration of DGAVP on recall of prose passages 24 h later (Beckwith et al., 1995); for related results (Fehm-Wolfsdorf et al., 1984b). Yet, these results contrast with findings of a recent study in our laboratory (Pietrowsky et al., 1998) which was designed to investigate the effects of VP on sleep-associated memory formation. During the acquisition phase before nocturnal sleep, the subjects learned a list of paired word associates, until a criterion of 60% correct words were recalled upon cued recall. After learning, subjects received either placebo or VP (40 IU, intranasally) and were allowed to sleep for 3 h. A second group of subjects was tested with identical conditions except that they were kept awake during the 3-h retention period. VP improved recall especially after the period of sleep, suggesting a supportive influence on consolidation when there is minor interference. Notably, this conclusion receives support also from studies showing a protective effect of VP against the memory impairment induced by electroconvulsive therapy (e.g. Mattes et al., 1990).

Type of memory encoding

While a considerable body of data indicates that VP is able to improve memory encoding, it can be asked whether this effect pertains specifically to a certain subtype of encoding. Enhanced consolidation could be a consequence of a strengthening of memory traces as achieved, for example, by mere rehearsal. Alternatively, enhanced consolidation can be achieved by encoding the target representation into a more dense and elaborated network of associative connections which would facilitate access to the target representation. Comparing recall and recognition of abstract versus concrete words, the work of Bruins (1991) and Bruins et al. (1995) provided some clues, suggesting that DGAVP influences both processes. Compared with abstract words, concrete words (e.g. dogs) are easier to recall which was explained by dual storage of the representations in an imagery system and a verbal store (e.g. Bower, 1972). Compared to abstract words, representations of concrete words are presumably characterized by a greater number of associative connections to other strong representations in LTM. Especially after subchronic treatment, DGAVP improved both indicators of memory for abstract as well as concrete words (Bruins, 1991). Interestingly, for concrete words, the effect concentrated on recall whereas for abstract words, recognition performance was enhanced. Thus, DGVP appeared to enhance the prevalent type of memory encoding respectively: i.e. the positive effect on recall of concrete words could well reflect a supportive influence of DGAVP on the formation of associative connections in LTM, whereas the positive effect on recognition of abstract words is likely to reflect a stronger engravement of representational memory traces.

A supportive effect of VP on the development of associative links during consolidation is suggested also by examinations of memory for sentences and stories. These tasks particularly promote the forming of associations and have repeatedly indicated an improved performance after VP (Posmurova et al., 1983; Till and Beckwith, 1985; Beckwith et al., 1984, 1987, 1990b), although in one study no effect emerged (Bruins, 1991). Most remarkable are the findings of Beckwith et al. (1987) indicating a dependence of recall of narrative prose on the importance of information. In the study, recall of prose was quantified by means of 'idea units' at different levels of importance. The men treated with DDAVP recalled more idea units at both high and intermediate levels of importance while no such effect could be confirmed for 'low important' information. We assume that an essential aspect of hippocampal function is to enhance activation of neural representations predominating on the basis of their biological significance and novelty, and thereby to selectively facilitate encoding of these representations into LTM (e.g. Vinogradova, 1975; Tulving et al., 1994). In this context, the findings of Beckwith et al. (1987) would point

to a supportive effect of VP on this 'enhancer' role of the hippocampus and related structures. This suggestion appears to be corroborated also by unit recordings from hippocampal slices (e.g. Chepkova et al., 1995).

Mismatch, P3 and orienting

Event-related brain potential responses (ERPs) and the spontaneous EEG activity have been used in the investigation of vasopressinergic effects, because these neurophysiological measures were deemed to reflect the central nervous influences of the peptide more directly than behavioral measures. In conjunction with appropriate tasks ERPs are sensitive indicators of the various aspects of the brain's information processing. The timed sequence of potential deflections comprising the ERP response reflect stimulus processing at different stages of early preconscious sensory integration of stimulus features and of later STM processes of stimulus evaluation and memory encoding. The emergence of changes in the ERP after VP sometimes depends on seemingly slight variations of the task paradigm. This is not surprising considering that such variation can induce distinct changes in the way the task is coped with and the associated component structure of the ERP. Nevertheless, a considerable number of studies have reported on an enhancing effect of VP on the N2 and P3 component complex of the ERP. Interestingly, both of these components have been linked to hippocampal function and, in this context, to the processing of biological significant, novel and unexpected events (e.g. Johnston et al., 1986; Donchin and Coles, 1988; Näätänen, 1988, 1992).

A major subcomponent of the N2 complex is the mismatch negativity (MMN) which emerges around 150 ms post-stimulus and is considered a correlate of the processing of stimulus 'deviance'. MMN has been most thoroughly investigated in the auditory modality, where it is elicited by a change in the physical characteristics of a stimulus presented in a repetitious series of others. It increases in amplitude with increased physical deviance from the repetitive standard stimuli and occurs also after a stimulus omission. It can be recorded independently of whether the stimuli are attended or ignored. Hence, MMN is considered a sign of the automatic, preconscious detection of stimulus change. The MMN reflects activity of neuron populations within the auditory cortex and possibly also in the frontal cortex (Hari et al., 1984; Giard et al., 1990; Sams et al., 1991). Moreover, in association with the early cortical MMN a distinct negative potential shift has been recorded from the dorsal hippocampus of cats (Csépe, 1995) presumably reflecting a neocortico-hippocampal dialogue subserving the evaluation of such features as the novelty or familiarity of a stimulus which eventually determine whether a stimulus will be encoded for memory (see also Tulving et al., 1994). Unattended stimuli invoking MMN exceeding a certain threshold amplitude, are able to trigger a switch of attention thereby attracting processing capacities from STM. In this case, MMN is followed by a positive deflection termed P3a which is a subcomponent of the late positive complex (LPC). Aside from P3a, the LPC includes also the P3 proper which develops around 280 ms post-stimulus and is typically evoked by task relevant stimuli which require a specific response. P3 amplitude is negatively correlated with the probability of a stimulus and increases with increased informational content and emotional valence of a stimulus (Johnson, 1986; Johnston et al., 1986). As a sign of conscious processing within STM it has been related to an updating of the model of the task situation residing STM (Donchin and Coles, 1988). Most important is that P3 amplitude also reflects the strength of memory encoding since several studies have demonstrated that P3 amplitude to a stimulus during learning is positively correlated with later recall (e.g. Karis et al., 1984; Fabiani et al., 1986; Paller et al., 1988). Source localization in animals as well as in humans revealed major contributions of the hippocampus formation and of the temporo-parietal cortical junction to the P3 genesis (Halgren et al., 1980; Knight et al., 1989).

VP and ERPs

ERP changes after VP have been extensively studied in the authors' laboratory (Table 2). One of the first studies adopting a classical attention

Table 2
Summary of ERP studies[a]

Study	N/sex design	Dose	Effects
Timsit-Berthier et al. (1982)	26/m within	14 IU LVP i.n.	Contigent negative variation ↑ N1-P2 Ø
Born et al. (1986)	5/m, 12/f group	3 × 20 IU LVP i.n. 48, 24, 1 h prior to test	MMN ↑ Nd Ø
Born et al. (1987)	17/m within	40, 80 IU VP i.n.	MMN ↑ P3 ↑ Nd Ø
Fehm-Wolfsdorf et al. (1988)	45/m within	3 × 20 IU VP i.n. 48, 24, 1 h prior to test	N1-P2 ↑
Pietrowsky et al. (1989)	13/m within	3 × 20 IU DGAVP i.n.	N2/MMN ↑
Naumann et al. (1991)	20/m, 20/f within	3 × 10, 3 × 20 IU DGAVP i.n. 48, 24, 1 h prior to test	P3 ↑
Pietrowsky et al. (1991)	12/m within	0.15, 0.5 IU VP i.v.	Auditory brainstem potentials Ø
Dodt et al. (1994)	50/m group	3 × 20 IU VP i.n. 22, 12, 1 h prior to test	N2/MMN ↑ N2-P3 ↑
Pietrowsky et al. (1996)	15/m within	20 IU VP i.n. 0.025, 0.1, 1.5 IU VP i.v.	P3 ↑ (after i.n. administration)

[a] Sex: m, male; f, female. Design: within, subject comparison; group, design. i.n., intranasally, i.v., intravenously. For AVP and LVP 1 IU is about 2.5 μg, for DGAVP 1 IU corresponds to 100 μg. MMN, mismatch negativity; Nd, negative displacement; ↑, enhanced amplitude; Ø, no effect.

task, yielded an effect of VP on both MMN and P3a (Born et al., 1987). In the task, series of tone pips were concurrently presented to the subject's right and left ear (Hillyard et al., 1973). The series in each ear consisted of frequent standard pips and some rare deviant tone pips randomly interspersed among the standards. The subject had to selectively attend to the stimuli in one ear at a time, and to count the rare deviant pips ('targets') therein, but to ignore all stimuli of the contralateral ear. VP administered intranasally at doses of 40 and 80 IU enhanced the MMN to deviant stimuli of the ignored ear. The effect was much stronger for the low than high dose of VP and was also distinctly more pronounced in the beginning of a tone series than in the second half. The latter finding could indicate that stimulus 'novelty' is of particular relevance for the enhancing effect of VP on MMN. At the same time, ERPs to the deviant target pips of the attended ear after VP displayed an enhanced positive potential 180–360 ms post-stimulus over fronto-central cortical areas which, in addition, appeared to be sensitive to habituation. The pattern was suggestive for an action of VP on P3a rather than classical P3.

The enhancing effect on MMN following VP confirmed those of a foregoing study in monocygotic twins (Born et al., 1986). Notably, both studies did not indicate an effect of VP on ERP signs of selective attention. Comparing the ERPs to the standard pips of the attended ear with those in the ignored ear typically reveals a slow negative potential displacement in the ERP to the attended pips (Nd, also termed processing negativity) which reflects the selectively increased processing capacities allocated to the attended ear stimuli (Elbert and Rockstroh, 1987; Näätänen, 1992; Hillyard et al., 1995). The underlying process of focusing attention has been ascribed to an interaction primarily between prefrontal cortical and thalamic structures, and apparently is not a primary target of VP actions.

Enhanced amplitudes of N2/P3 related ERP components following VP were also observed in the context of other task paradigms. In a classical oddball task situation with target pips requiring a button press response, occasionally interspersed among frequent standard pips, VP increased the more anteriorly distributed N2 as well as the N2-P3 amplitude difference over parietal cortical areas (Dodt et al., 1994). However, in the group of elderly of this study which exhibited the typical age-related decrease in P3, VP enhanced N2 amplitude, only. Thus, the peptide failed to improve the ERP sign most characteristic for the age-related impairment of stimulus processing. Using a more elaborate stimulus-mismatch paradigm in young men, Pietrowsky et al. (1989) were able to unravel a clear-cut enhancing effect of DGAVP on MMN. The task paradigm was comprised of a series of standard pips, among which two different kinds of deviants tones were randomly interspersed, i.e. 'proximates' and 'extremes'. Compared to the proximates, extremes occurred less frequently and deviated more extremely in pitch from the standards. Consequently, MMN to these tones was maximal. The increase in MMN after DGAVP was particularly prominent in ERPs to these tones. It was more pronounced when the subjects listened to the tones than when reading a magazine during tone presentation.

Two further studies provided evidence for a focus of VP effects on P3 (Naumann et al., 1991; Pietrowsky et al., 1996). Interestingly, in both studies the effect did not dominate over parietal cortical areas where the classical P3 predominates, but was most pronounced over the frontal scalp. In the study of Naumann et al. (1991) the classical 'oddball' P3 remained unaffected by VP possibly due to the elevated target frequency chosen in this experiment. However, P3 was strongly enhanced in an encoding task in which the subject had to estimate the number of letters of visually presented words (adjectives). The words each of which was presented only once, induced a late frontal positive potential shift which was dramatically enhanced following VP at the dose of 3 × 20 IU, and still significantly enhanced at the lower dose of 3 × 10 IU. Moreover, the effect appeared to be more distinct for adjectives with negative or positive meaning than for neutral ones. Remarkably, when unexpectedly asked to recall the words, subjects following VP also displayed an improved memory performance.

Together, these ERP findings provide an important clue to the psychophysiological mechanisms of VP actions in humans. The coherent enhancement of N2-related negativity and of P3 over the anterior cortex after VP in different task paradigms, as well as the apparent sensitivity of these effects to stimulus novelty, expectancy and valence fits with the hypothesis of VP exerting its action primarily on hippocampal and closely connected structures. The hippocampus has been implicated to be a central structure in a memory encoding network determining which information is to be encoded for long-term storage (Tulving et al., 1994). The underlying mechanism of this action could be based on an amplification of the signal-to-noise ratio within ongoing stimulus processing. Through this influence, neural representations distinctly preactivated, gain further dominance to the exclusion of others. VP thereby may facilitate the preferential processing of novel, unexpected and salient inputs and (once in STM) their encoding for memory storage. This view comes close to a suggestion by Strupp and Levitsky (1985) and Bunsey et al. (1990) that VP release may 'stamp in' the preceding and ongoing neural events, such that the memory traces most relevant for environmental adaptation will be more easily remembered.

A hippocampal site of vasopressinergic actions enhancing the processing of novelty and mismatch would also fit findings, indicating an increased stimulus-induced arousal after VP. It has been proposed that during encoding of information, the hippocampus mediates a tonic inhibitory control over arousing structures of the brain-stem reticular formation which is withdrawn upon orienting to novel signals (Redding, 1967; Vinogradova, 1975). Accordingly, enhanced hippocampal processing of novelty and mismatch would be expected to diminish this inhibition thereby increasing arousability. Fehm-Wolfsdorf et al. (1984a, 1988) tested effects of VP on ERPs in a rather boring auditory vigilance task requiring the subject simply to count a repetitively presented tone. Due to the high familiarity of the tone, N2 and P3 cannot be assessed

with this kind of task. However, VP distinctly enhanced the earlier N1/P2 component complex which is a sign of diffuse stimulus-induced arousal mediated via collateral activation of the brain-stem reticular formation (Näätänen and Picton, 1987). A similar mechanism may underly observations of reduced EEG alpha-power (e.g. Fehm-Wolfsdorf et al., 1985) and an increased amplitude of the contingent negative variation (CNV) in the ERP after administration of VP (Tecce, 1972; Timsit-Berthier et al., 1982). While these findings suggest an effect on extraleminiscal brain-stem arousing structures, transmission within specific lemniscal brain-stem pathways (assessed by auditory brain-stem potentials) remained unaffected by VP (Pietrowsky et al., 1991).

Contrasting with the proposed view of enhanced novelty processing after VP, Baltissen et al. (1991) failed to demonstrate an effect of VP on the skin conductance and heart rate components of the orienting reaction. These negative findings are difficult to integrate, but could be related to the relatively inert nature of these peripheral autonomic responses, as compared to the ERP indicators of orienting. Likewise, indicators of autonomic nervous activity, such as blood pressure and heart rate, consistently failed to reveal any increase in the tonic arousal level (e.g. Beckwith et al., 1982; Posmurova et al., 1983; Sahgal et al., 1986; Pietrowsky et al., 1991).

Attention

The two principle aspects of attention are its selectivity (i.e. the filtering of certain stimulus inputs to the exclusion of others) and its maintenance, i.e. vigilance. ERP signs of attention have been discussed above. Studies did not indicate an effect of VP on the Nd correlate of selective attention (Born et al., 1986, 1987). With regard to vigilance, signs of an increased arousal were occasionally observed following VP which may support sustained attention. The effect was assumed to be secondary to an increased hippocampal processing of novel, unexpected and salient events.

Enhanced processing of such stimulus aspects is supposed to facilitate memory encoding. Yet, further STM operations could be expected to benefit from this action, including processes of stimulus classification and response selection. Various tests were employed to elucidate the effects of VP on these STM functions at different levels of complexity. Although showing little sensitivity to VP, different versions of paper-and-pencil cross-out tests (e.g. the Bourdon test) have been used most frequently. These tasks require to cross out as fast and as accurately as possible a certain symbol (e.g. letter, digit) randomly interspersed among a series of other more or less similar symbols. DDAVP did not affect performance on an extended version of such a test in which a total of 78 000 digits were to be scanned (Sahgal et al., 1986). Laczi et al. (1982, 1983) reported on an improved performance on the Bourdon-test following DGAVP. However, this study did not adequately control for practice effects. In another study (Snel et al., 1987) after DGAVP the error rate on the Bourdon test remained unchanged. However, the number of errors which were acutely corrected by the subject was reduced. The authors related this outcome to a slower but 'more attentive and thus more accurate' way of performance following DGAVP.

A more complex stimulus classification is tested in the Sternberg paradigm (Sternberg, 1969). The subject is first presented with sets of digits or letters which on different trials are of different size (including 1, 2, 3 or 4 symbols). After memorizing the set, a series of symbols is presented and the subject has to respond as quickly as possible whenever a symbol of the set is presented. Reaction time increases with increased set size. The slope of this function is used to indicate memory scanning, whereas the reaction time at set size 1 indicates the speed of perceptual encoding and motor programming. One study (Beckwith et al., 1983) reported on shortened reaction times after DDAVP independently of the set size suggesting speeded perceptual encoding and motor programming. In another study (Nebes et al., 1984) the accelerating effect of DDAVP on reaction times was most pronounced for large set sizes suggesting an effect on memory scanning. A third study (Bruins, 1991) failed to disclose any effect of DGAVP. Similarly ambiguous results were revealed for the Stroop–Color–Word test (Stroop, 1935). In the interference condition of this test, the

subject is presented with a list of color words written in a different color than the meaning of the words. Instead of reading the word, the subject has to name the color as fast as possible. Improved performance on this condition as observed by Fehm-Wolfsdorf et al. (1984a) is suggestive of an improved encoding selectively of the stimulus features relevant for the task. Bruins et al. (1992) and Bruins (1991) observed no effect of DGAVP with this test, and Beckwith et al. (1990a) even revealed signs of impaired performance in women treated with DDAVP.

Processes of response selection were also investigated at different levels of complexity. Carter et al. (1991) observed a speeded reaction time following DDAVP in a simple warned reaction task as well as in a 'complex movement task'. On the latter task three different movement sequences had to be perfomed in response to a green, red or blue light. In contrast, in a comparable task Bruins (1991) failed to reveal any effect of DGAVP. A most interesting test represents the dual task paradigm adopted by Jennings et al. (1986) to examine effects of intranasal DGAVP. The subjects were told to solve mental arithmetic tasks (primary task) but simultaneously they should keep track of occasionally presented light flashes which were to be answered with a fast button press (secondary task). DGAVP lengthened reaction time in the secondary task which was deemed to be less important than the primary task. This finding may be taken to suggest that VP weakens STM processing of less relevant stimuli to the advantage of the most relevant input. Unfortunately, a beneficial effect of DGAVP on the primary task of mental arithmetic could not be demonstrated. However, this failure fits with observations from other studies (e.g. Bruins, 1991), and probably reflects the fact that mental arithmetic performance only rather crudely reflects STM operations of thought.

In this regard, a concept formation task proved to be more sensitive (Beckwith et al., 1982). On each trial of the task, two of various different colored geometrical forms were presented (e.g. red, black, blue, and white circles, squares, crosses and triangles). The subject was reinforced whenever he chose the figure matching with the target concept (e.g. the color 'red'). Following DDAVP, the men needed less trials to identify the correct concept. Also, when after concept identification, the reinforcement contingencies were changed and the choice of another concept (e.g. 'triangle') was reinforced, the men after DDAVP needed less trials to identify the new target concept. Interestingly, concept formation performance has been shown to be accompanied by an increased EEG theta rhythm most likely generated in septo-hippocampal structures (Gerbrandt et al., 1978; Lang et al., 1988).

Overall these findings indicate that VP is, indeed, able to enhance STM functions not directly related to memory encoding. The exact conditions triggering the emergence of such effects are presently obscure. Effects of VP seemed to be somewhat more consistent in complex than simple tasks. Promising results were obtained with tests of thinking such as the concept formation task which demand the inductive formation of complex associative connections. Also, in light of the widely used reaction time measures it has to be cautioned against the presumption that VP effects on STM operations always express themselves in a change in the speed of reactions. This is underlined also by the many findings indicating only loose correlations between reaction time and amplitude measures of MMN and P3 which more sensitively reflect effects of VP (e.g. Verleger, 1997).

Arousal and mood

In agreement with others, in a previous review we have proposed that VP acts on memory and attention indirectly via an enhancing influence on non-specific central nervous arousal (Sahgal, 1984; Fehm-Wolfsdorf and Born, 1991). In the case of systemic administration, VP was supposed to increase arousal through peripheral changes which in turn are signaled to the brain's arousal structures. However, the intranasal administration of VP which is highlighted here, did not induce any noteworthy changes in blood pressure, heart rate or pituitary–adrenal activity in any of the experiments discussed, which makes a peripheral mediation of the effects unlikely. Following intranasal administration, VP most likely exerts its influence by entering the cerebrospinal fluid. Hence, a direct effect on arousing structures via VP receptors in the brain-

stem is conceivable, although evidence for this kind of an effect is, indeed, weak.

Apart from ERP and EEG recordings (discussed above) also self-report measures occasionally indicated arousal-related changes after VP such as decreased feelings of 'deactivation' and 'fatigue' (Pietrowsky et al., 1991). In another study, subjects after DGAVP felt more 'moody', 'elated' and 'conscientious' (Snel et al., 1987). However, in the majority of studies, VP lacked any influence on experienced feelings of activation and mood (e.g. Beckwith et al., 1982; Sahgal et al., 1986; Bruins et al., 1990, 1992; Pietrowsky et al., 1992). Effects of VP on self-report measures may in fact be rather sensitive to ambient characteristics of the experimental setting, and increases in arousal after VP seemed to occur mainly in settings which included boring tasks and were apparently less demanding. However, overall the size of these effects and their inconsistent occurrence argue against a primary effect of VP on arousal. This impression is further enforced when effects of VP on neurophysiological signs of arousal are compared with those of drugs with a clearly more potent arousing action, such as amphetamines (Saghal, 1987; Born and Debus, 1997).

Nevertheless, an influence of VP on arousal was presumed to explain some of the inconsistencies in the changes in memory. Specifically, an increase in arousal after VP (shifting arousal towards levels more or less optimal for performance) has been taken to explain a dependency of VP effects on memory on the time of day at testing and on circadian rhythm (Pietrowsky et al., 1988; Bruins, 1991). While these assumptions remain speculative, a factor of greater importance than arousal for a circadian modulation of VP effects could be the endogenous glucocorticoid concentration at testing which in humans is distinctly higher during the morning than afternoon hours. Glucocorticoids have been shown to impair declarative memory (e.g. Kirschbaum et al., 1996; Plihal et al., 1998). This effect could well counteract the improving effect of exogenous VP on memory, especially, when it is considered that one of the multiple actions of VP is to enhance expression of hippocampal corticosteroid receptors (Veldhuis and De Kloet, 1982; Sapolsky et al., 1984).

Sleep

An issue closely related to the level of arousal and plasma corticosteroids concerns the effects of VP on sleep which have been examined in healthy humans in five studies so far (Timsit-Berthier et al., 1982; Snel et al., 1987; Born et al., 1992; Perras et al., 1997, 1998). In none of the studies, acute or subchronic administration of VP, LVP and DGAVP exerted any effect on subjective sleep quality. Continuous intravenous infusions of VP throughout the night, at doses of 0.33, 0.66 and 0.99 IU/h, revealed a dose-dependent decrease in rapid eye movement (REM) sleep. Less consistent effects were an increase in stage 2 sleep and in time awake. An increase in stage 2 sleep was observed also after intranasal administration of 14 IU LVP (Timsit-Berthier et al., 1982). Substantial changes in plasma levels of cortisol and blood pressure were not observed in these studies after VP. Although the increase in stage 2 sleep and in time awake after VP were reminiscent of an arousing effect, compared with the reduction in REM sleep, these changes were clearly less consistent. Since also, clear-cut effects on slow wave sleep were missing, a primary effect of VP on sleep via arousing structures seems less likely. Alternatively, an action secondary to an effect on REM sleep generating mechanisms has been discussed. Studies in Brattleboro rats indicated a regulatory role of VP for the hippocampal theta rhythm prevailing during REM sleep, and in normal rats continuous intracerebroventricular infusion of the peptide reduced time spent in REM sleep (Urban and DeWied, 1978; Kruisbrink et al., 1987).

Also, effects of VP on sleep may involve an interaction with circadian oscillators. This view finds support from results of recent studies exploring the effects of subchronic treatment with VP on sleep in healthy elderly (Perras et al., 1997, 1998). Sleep in elderly is characterized by diminished slow wave sleep and REM sleep and an increased frequency of awakenings. In the elderly of those studies, 2 × 20 IU VP, administered intranasally before bedtime and after morning awakening daily over a period of 3 months distinctly increased total sleep time and time in slow wave sleep. In addition, a less distinct increase in REM sleep

was observed clearly contrasting with the findings after acute VP administration in young subjects. For the beneficial effect of VP in aged alternative explanations were put forward. An action via stimulation of hippocampal corticosteroid receptor expression (Sapolsky et al., 1984) must be taken into consideration in light of evidence that activation of limbic mineralocorticoid receptors can result in considerable increases in slow wave sleep (Born et al., 1991). Alternatively, subchronic treatment with VP may compensate for an age-related decrease in the volume and number of cells expressing VP in the nucleus suprachiasmaticus (Hofman and Swaab, 1994; Swaab, 1995). Considering the predominant role of this nucleus as pacemaker of circadian rhythm, it is conceivable that the improved sleep, and in particular the overall increased time asleep during the night in elderly, resulted from an enhancing influence of the VP treatment on the amplitude of the sleep–wake rhythm.

Conclusions and perspectives

In rats, VP exerts its principle behavioral action on memory formation via the septo-hippocampal system, along with connected structures. The research reviewed here, is in agreement with the notion that the same structures represent the essential mediators of behavioral changes seen after VP administration in healthy humans. Evidence exists that the intranasal administration of VP in humans is functionally equivalent with the intracerebroventricular administration employed in animals. The intranasal administration of VP induces substantial increases in CSF concentrations of VP while in all experiments discussed, peripheral hormonal effects such as vasopressor actions and the activation of pituitary-adrenal secretory activity remained negligible. Fortunately, the great majority of studies in healthy humans relied on the intranasal route of VP administration.

Although methodological shortcomings limit the validity of some experiments, the majority of studies hint at a beneficial effect of VP on memory function which agrees with the memory hypothesis proposed by De Wied on the basis of animals research (De Wied et al., 1988). The positive findings in humans concerned exclusively functions of declarative memory which is the type of memory essentially relying on hippocampal function. Procedural memory formation was not affected. The data suggest that the effect of VP centers on the encoding process for declarative memory, which refers to the transfer of information from STM into long-term storage, and is linked to specific operations in STM that enable a strengthening of the respective neural representation and of its associative links to other representations in LTM.

Studies using ERP indicators of stimulus processing point to an enhancing effect of VP on those operations linked to the novelty, unexpectedness and salience of a stimulus. Novel and salient stimuli can evoke MMN and a P3a which appeared to be the ERP components most sensitive to the influence of VP. The genesis of these components involves an essential contribution of hippocampal activity. Moreover, since P3 related positivity has been also shown to predict later recall of an event, the enhancement of this potential after VP agrees with the findings of improved retrieval following administration of the peptide. Therefore, it is tempting to propose that the facilitating effect of VP on memory encoding results from enhanced septo-hippocampal processing of aspects related to the novelty, unexpectedness and salience of an event. Behavioral tests gave some indications that apart from memory encoding, VP could improve further aspects of STM processing including stimulus evaluation and response selection. The exact conditions for the emergence of these effects are still obscure, although they may also depend on such factors as the novelty and complexity of a stimulus input. Mechanisms regulating voluntary focusing of attention (selective attention) and vigilance do not appear to be a primary target of VP actions, although increased processing of novel and rare stimuli may result in a greater potency of these stimuli to attract attention.

It has been proposed that the peptide acts mainly by enhancing central nervous arousal. Although this type of effect cannot be entirely abandoned, it is to note that effects of VP on ERP and EEG signs of arousal are of moderate size only, and appear to be less consistent than those on memory. Consider-

ing evidence that the hippocampus and related structures exert a tonic inhibitory control over arousal mediating structures of the mid-brain reticular formation which is withdrawn during orienting, signs of enhanced arousal after VP may well result from its action on septo-hippocampal structures. A similar mechanism could underly the acute decrease in REM sleep after VP. In contrast, the improvement of sleep after subchronic treatment with VP may involve an action on the hypothalamic nucleus suprachiasmaticus.

Although some outlines of a profile of actions of VP on human brain function and behavior can be recognized on the basis of the present data, important experiments remain to be done. Of course, the pharmacological conditions for the effects of VP in humans need further elaboration, including the mechanisms of intranasal VP administration. Moreover, some studies revealed a dependency of size (e.g. Born et al., 1987) and direction (e.g. Beckwith et al., 1990b) of VP effects on the dose administered. Likewise, size (Bruins, 1991) and direction (Perras et al., 1998) of the VP effect may depend on whether acute or chronic actions of the peptide are considered. Evidence exists also that males are more sensitive to the beneficial effects of VP than females which matches with observations of a gender-specific organization of the limbic–hippocampal VP system (e.g. Beckwith et al., 1983, 1990a; Bruins, 1991). Finally, it has to be considered that apart from VP, the hippocampus is the target of many other neuroendocrine substances, such as glucocorticoids, which interact and potentially interfere with the effects of VP.

Comparing findings in healthy humans with those in animals, it has to be recognized that that across the numerous tasks employed the effects of VP appear to be less robust than those in rats. Therefore, research would benefit profoundly from the development of a task paradigm which allows the effects of VP to be discriminated in humans as clearly as passive avoidance tasks do in rats. From the data reviewed here, one would expect that such a task involves the presentation of novel, rare and salient events which are consciously processed to be encoded for memory. Yet, these features may not be the only determinants.

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Subject Index

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