PROGRESS IN BRAIN RESEARCH

VOLUME 170

ADVANCES IN VASOPRESSIN AND OXYTOCIN — FROM GENES TO BEHAVIOUR TO DISEASE

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AMSTERDAM – BOSTON – HEIDELBERG – LONDON – NEW YORK – OXFORD
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Elsevier
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK
Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands

First edition 2008

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British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

ISBN: 978-0-444-53201-5
ISSN: 0079-6123

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Printed and bound in Hungary

08 09 10 11 12 10 9 8 7 6 5 4 3 2 1

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Preface

The roots of neurohypophysial hormone research can be traced back to the end of the 19th century, when Ramon y Cajal, in 1894, described a neural pathway from the supraoptic nucleus of the hypothalamus to the posterior pituitary, and Oliver and Schäfer demonstrated blood pressure effects of neurohypophysial extracts in 1895. Thirty to forty years later, the German biologists Ernst and Berta Scharrer postulated the existence of specialized neurons in the hypothalamus of invertebrates, as well as vertebrates, which formed and secreted cytoplasmic vesicles, comparable to those found in peripheral secretory cells. Finally, in 1951 Wolfgang Bargmann and Ernst Scharrer demonstrated that vasopressin and oxytocin are synthesized in neurons of the hypothalamus, specifically within the supraoptic and paraventricular nuclei, irrefutably establishing the existence of the hypothalamo-neurohypophysial system. These neurons are characterized by their long axonal processes projecting to the posterior pituitary (or neurohypophysis as a synonym), where the nerve terminals form close contact zones with blood capillaries. Vasopressin and oxytocin are transported within neurosecretory vesicles along these axons to the posterior pituitary and are secreted into blood circulation upon appropriate stimulation.

Ever since this system was described in the fish brain, this well-defined and compact arrangement of relatively large neurons at the base of the brain has served as the gold standard in neuroendocrinology and neuroscience. In 1952, Vincent du Vigneaud isolated the neurohypophysial peptides and, subsequently, he and independently Roger Acher elucidated their chemical structure as a prerequisite for their synthesis. As a logical consequence of these discoveries, the physiological functions of vasopressin and oxytocin were revealed in great detail, i.e. the antidiuretic and cardiovascular effects of vasopressin, and the promotion of uterine contractions and of milk ejection by oxytocin, using posterior pituitary extracts or later the synthetic neuropeptides. In concert with the hormones released from the hypothalamus to regulate the adenohypophysial functions, the neuropeptides of the hypothalamo-neurohypophysial system mediate the functional impact of the brain on complex processes related to physiological homoeostasis, metabolism, growth, sexual and reproductive functions and stress coping to name but a few.

A new era of vasopressin and oxytocin research started in the early 1970s, when David de Wied described the first behavioural effects of neurohypophysial neuropeptides and their fragments, thereby synergizing the fields of neuroendocrinology and behavioural neuroscience. Shortly after, the description of neurophysin-containing pathways, mainly originating in the hypothalamus and projecting to extra-hypothalamic targets, added significant weight to a novel and emerging concept of brain vasopressin and oxytocin systems, which were independently regulated from the activity of the hypothalamo-neurohypophysial system.

Harold Gainer has described the hypothalamo-neurohypophysial system as a "veritable Rosetta stone for neuroendocrinology and neuroscience". Indeed, outstanding neurobiological discoveries have been founded on this neurobiological model system with big (i.e. magnocellular) neurons, which are easily accessible, identifiable by electrophysiological, immunohistochemical or in situ hybridization techniques, and localized in compact and relatively homogenous nuclei. Thus, over the last 40 years, important insights have been gained into the bursting pacemaker activity of neurosecretory neurons, the stimulus-secretion coupling, the organization of neuropeptidergic pathways and local release patterns within the brain, neuronal–glial interactive plasticity, the development of neuropeptide receptor antagonists and,

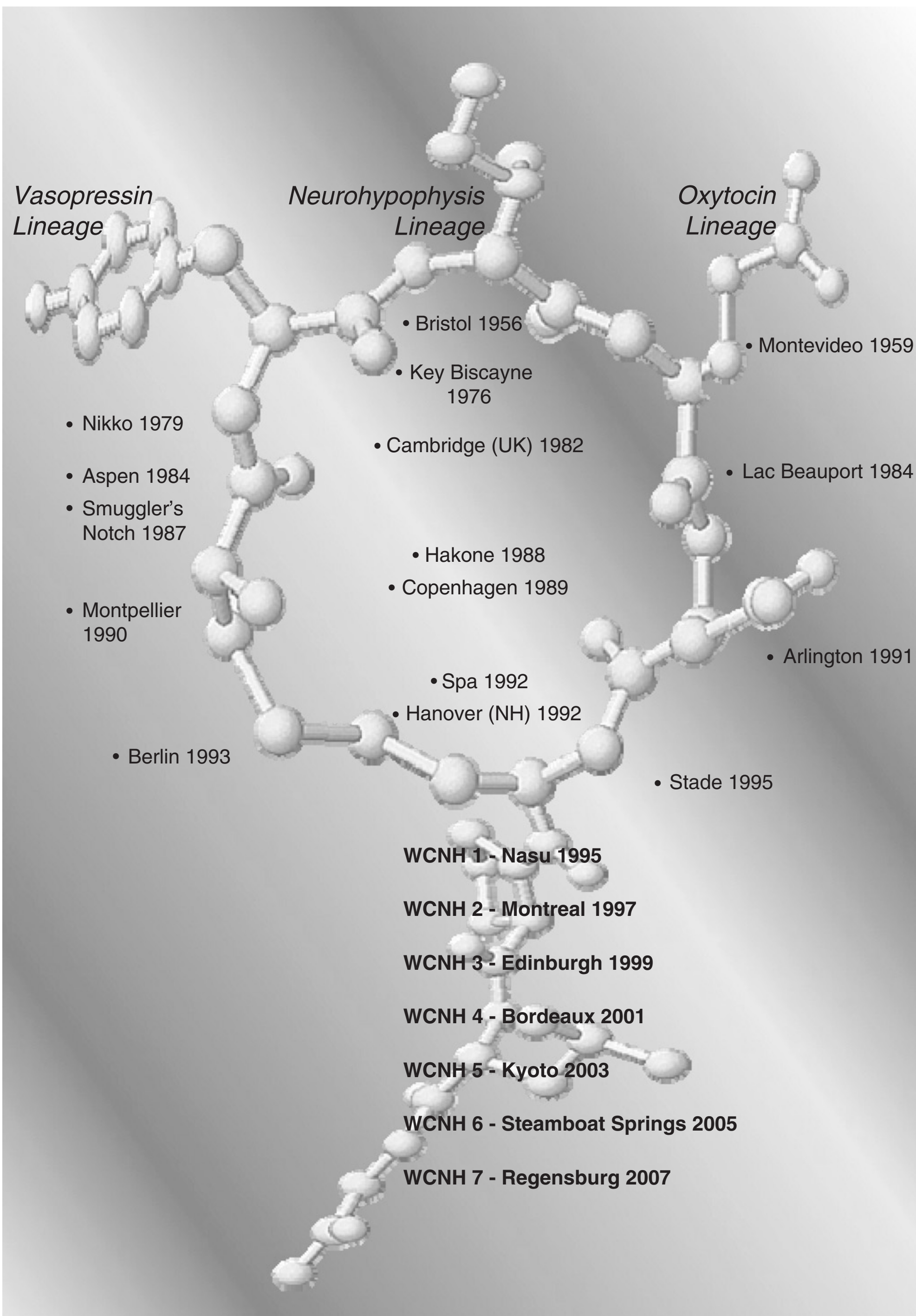

Fig. 1. "Molecular pedigree" of neurohypophysial hormone conferences. This retrospective view is based on a previous WCNH preface (Zingg, H.H., Bourque, C.W. and Bichet, D.G. (Eds.) (1998). Vasopressin and Oxytocin: Molecular, Cellular, and Clinical Advances. Plenum Press, New York, p. vi) and incorporates inputs from M. Manning and E. Frank. The underlying oxytocin molecule has been adapted from www.medicalcomputing.net.

importantly, a broad variety of behavioural actions, just to mention a few. Moreover, especially in the last two decades, molecular and cellular mechanisms of oxytocin and vasopressin synthesis, processing, release and neuronal feedback actions and the pharmacological and genetic manipulations of these systems could be significantly advanced. Both neuropeptides continue to attract intense attention due to the discovery of the amazing array of behavioural functions they mediate.

Central oxytocin and vasopressin modulate numerous social behaviours, such as maternal care and aggression, sexual behaviour (oxytocin), pair-bonding, social memory (vasopressin and oxytocin), face recognition, the positive health consequences of social support and human trust (oxytocin). Moreover, both neuropeptides regulate the behavioural and neuroendocrine responses to stress in an opposite direction. Thus, while neuropeptides such as vasopressin and corticotropin-releasing factor (CRF) could represent Scylla and Charybdis in behavioural regulation, oxytocin, like Circe, would mediate anxiolytic and pro-social influences, beneficial to relief, reproduction and love. These discoveries and suggestions position these neuropeptides amongst the most promising neuromodulator/neurotransmitter systems of the brain for the treatment of numerous psychiatric illnesses, including anxiety-related disorders, social phobia, autism and postpartum depression.

The amazing scientific breakthroughs achieved regarding these neuropeptidergic systems over the last 50 years have been reflected in numerous international meetings on the neurohypophysis, or, separately, on vasopressin or oxytocin. The first such meetings took place in Bristol in 1956 and in Montevideo in 1959, respectively. It was only in 1995, that these separate scientific lineages were unified with the organization of the First Joint World Congress on Neurohypophysial Hormones (WCNH) in Nasu by our Japanese colleagues and friends based around Toshikazu Saito. Since then, the WCNH has taken place biennially, if possible on another continent, in another country organized by members of the ever-growing WCNH family. All these meetings have their individual scientific, social and geographic flavour. Each of them (listed in Fig. 1) is remembered by the participants in relation to their distinct scientific breakthroughs, thoughtful scientific preparations by the respective scientific and local organizing committees and last, but not least, pleasant socializing, thus examining the pro-social neuropeptide effects within our WCNH family.

In this line of scientific tradition, the VIIth WCNH took place in Regensburg, Germany, from 18th to 22nd September 2007 with 255 participating scientists from 32 countries. The Regensburg meeting followed the meeting in Steamboat Springs, USA, in 2005 organized by the team led by Celia Sladek. The organization of the next WCNH, the VIIIth, is in the competent hands of Yoichi Ueta and Yutaka Oiso and will take place in the city of Kitakyushu, Japan, from 4th to 8th September 2009.

Also, in continuation of the tradition of the WCNH, we aimed at producing a book published by Elsevier summarizing the most recent state of the art findings. Therefore, this volume covers vasopressin and oxytocin research in a broad scientific context, encompassing a wide variety of topics from basic neuroendocrinology including molecular, genetic and cellular aspects, to clinical neuroscience, including reproduction, cardiovascular and renal physiology and psychopathology. In this sense, the volume should be of vital interest to specialists and those not yet closely related to this field. Thus, our hope is that it will be acknowledged as the current "Bible of Neurohypophysial Hormones Research".

Inga D. Neumann
Rainer Landgraf
Regensburg and Munich, December 2007

Acknowledgements

The WCNH 2007 would like to thank the following sponsors whose financial support of the meeting was greatly appreciated:

- German Research Foundation (DFG)
- Sanofi Aventis
- Regensburger Universitätsstiftung Hans Vielberth
- Serono
- Organon
- Wyeth Research
- Ferring Pharmaceuticals
- Roche
- DRG
- VfU Verein der Freunde der Universität Regensburg
- German Society of Endocrinology (DGE)
- Novartis
- Charles River Laboratories
- Journal of Neuroendocrinology
- Behavioural Brain Research
- Stress

I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 1

Nonapeptides and the evolutionary patterning of sociality

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Abstract: Neuropeptides of the arginine vastocin (AVT) family, including the mammalian peptides arginine vasopressin (AVP) and oxytocin (OXT), comprise neuroendocrine circuits that range from being evolutionarily conserved to evolutionarily diverse. For instance, the functions and anatomy of the AVT/AVP projections to the pituitary (which arise in the preoptic area and hypothalamus) are strongly conserved, whereas the functions and anatomy of AVT/AVP circuits arising in the medial bed nucleus of the stria terminalis (BSTm) are species-specific and change rapidly over evolutionary time. Circuits arising in the BSTm mediate various affiliative behaviors and exhibit species-specific evolution in relation to mating system in mammals (monogamous vs. non-monogamous) and sociality in songbirds (gregarious vs. relatively asocial). In estrildid songbirds AVT neurons in the BSTm increase their Fos expression only in response to "positively-valenced" social stimuli (stimuli that normally elicit affiliation), whereas "negative" stimuli (which elicit aggression or aversion) produce no response or even suppress Fos expression. Relative to territorial species, gregarious species show: (1) greater social induction of Fos within AVT neurons, (2) a higher baseline of Fos expression in AVT neurons, (3) more AVT neurons in the BSTm and (4) a higher density of V_{1a}-like binding sites in the lateral septum. Furthermore, septal AVT infusions inhibit resident–intruder aggression, but facilitate aggression that is motivated by mate competition (an affiliative context). This functional profile of the BSTm AVT neurons is quite distinct from that of hypothalamic AVT/AVP neurons, particularly those of the paraventricular nucleus (PVN), which are classically stress-responsive. This is paradoxical, given that AVT/AVP projections from the PVN and BSTm likely overlap. However, despite this overlap, each AVT/AVP cell group should produce a distinct pattern of modulation across brain regions. Relative weighting of hypothalamic and BSTm nonapeptide circuitries may therefore be an important determinant of approach–avoidance behaviour, and may be a prime target of natural selection related to sociality.

Keywords: vasotocin; vasopressin; oxytocin; isotocin; evolution; social behaviour

Nonapeptides and the patterning of behaviour

Neuropeptides of the arginine vasotocin (AVT) family, the nine amino acid "nonapeptides", are important generators of behavioural diversity, perhaps more so than any other neurochemical systems. This is mainly due to the dramatic plasticity of socially-relevant nonapeptide circuits. Plastic responses to steroid hormones and/or photoperiod cues are commonly observed for nonapeptide systems (Goodson and Bass, 2001; De Vries and Panzica, 2006), and these responses

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DOI: 10.1016/S0079-6123(08)00401-9

promote temporal patterns of behaviour that are appropriate to the season, immediate social context and physiological state of the animal. Nonapetide circuits are likewise plastic in the evolutionary sense. This is particularly observed with respect to receptor distributions, which can vary qualitatively across species (Insel et al., 1991, 1994; Insel and Shapiro, 1992; Bester-Meredith et al., 1999; Goodson et al., 2006), thereby setting up dramatic species differences in behaviour.

On a much shorter timescale, the nonapeptides significantly influence context-dependent patterns of socially-elicited neural response. As addressed in the final section, different cell groups that produce a given nonapeptide may exhibit quite divergent (in some ways opposite) responses to social stimuli, and the projections of these cell groups may even converge onto some of the same targets. However, each population should produce a different *pattern* of modulation across behaviourally-relevant brain regions. Differential "weighting" of activity in basal forebrain networks is an important determinant of social behaviour output (Newman, 1999; Goodson, 2005); hence, the distinct patterns of release from the various nonapeptide cell groups could produce very different (even opposite) behaviour patterns, both within and between species.

Deep history of the nonapeptides

AVT is present in all non-mammalian vertebrates examined to date and is the ancestral nonapeptide form for all of the vertebrate nonapeptides (Acher and Chauvet, 1995; Acher et al., 1995). Given this basal position of AVT, the vertebrate nonapeptides are collectively referred here to as the "vasotocin family". However, AVT is structurally similar to a variety of invertebrate nonapeptides (Fujino et al., 1999), including a form in the nerve net of the freshwater hydra (*Hydra attenuata*), suggesting that the AVT-like nonapeptides are much more ancient than AVT itself and have an evolutionary history that dates back to the Precambrian era, more than 600 million years ago (Acher and Chauvet, 1995; Acher et al., 1995). Furthermore, recent analyses show that VT neurophysin neurons within neurosecretory brain regions of both annelids and fish express the same tissue-specific microRNA and same combinations of transcription factors. These neurons appear to serve as sensory-neurosecretory linkages and are likely among the most conserved elements of the neurosecretory brain (Tessmar-Raible et al., 2007).

The primary nonapeptides in vertebrates are shown in Fig. 1. As shown, AVT is the sole nonapeptide form in agnathans (jawless vertebrates). The AVT gene was duplicated in early fishes (i.e., early jawed vertebrates), approximately 450 million years ago, forming a second oxytocin (OXT)-like peptide. The most common OXT-like form in fishes is isotocin (IT), which is present in all teleosts (bony fishes) thus far examined. Sometime prior to the water-to-land transition, IT was replaced in the common ancestor of tetrapods by mesotocin, which is retained in crossopterygian

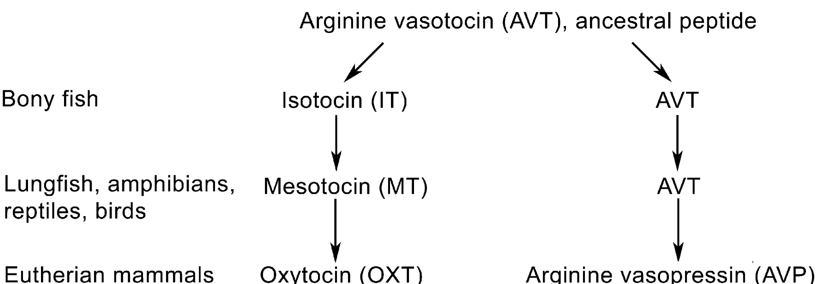

Fig. 1. Evolution of the vasotocin nonapeptide family in vertebrates (after Acher and Chauvet, 1995). Only main features are shown. Secondary duplications and other peptide forms are present in some vertebrate groups (e.g. marsupials and cartilaginous fish; see "Deep History of the Nonapeptides").

fish and all tetrapods except eutherian and protherian mammals, which express OXT. Despite these multiple modifications, AVT and OXT differ by only one amino acid. Evolution on the AVP side of the family has been even more conservative, with AVP replacing AVT in most mammals. AVT and AVP also differ at only one position (for review, see Moore, 1992; Acher and Chauvet, 1995; Acher et al., 1995; Moore and Lowry, 1998).

Most vertebrates possess two nonapeptide forms, including an AVP-like form and an OXT-like form. However, secondary duplications are present in a variety of groups, particularly the cartilaginous fishes, and some groups therefore express three or even four nonapeptides. At least six different OXT-like peptides are expressed in cartilaginous fish (OXT, glumitocin, valitocin, aspargtocin, asvatocin and phasvotocin), with one or two forms being present in any given species. Secondary duplications are also common in marsupials, which express three different AVP-like forms (AVP, lysipressin and phenypressin) in addition to both mesotocin and OXT. Most eutherians express only AVP and OXT, although lysipressin replaces AVP in pigs (Moore, 1992; Acher and Chauvet, 1995; Acher et al., 1995).

Evolution of central nonapeptide circuits: anatomical and behavioural basics

Evolutionary conservation in the structure of the nonapeptides, as described above, is substantially mirrored by conservative evolution in the locations of main cell groups in the brain. In all vertebrates, the AVP-like and OXT-like peptides are produced by populations of magnocellular and parvocellular neurons in the preoptic area (POA) and anterior hypothalamus (AH). Magnocellular neuron populations are present in the magnocellular POA of fish and amphibians; these are homologous to magnocellular nonapeptide neurons in the supraoptic nucleus (SON) of the hypothalamus of reptiles, birds and mammals. Parvocellular neuron populations are found in the parvocelluar POA of fishes and posterior POA of amphibians, which are homologous to parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus in amniotes (Moore and Lowry, 1998; Goodson and Bass, 2001). These cell groups project to the neurohypophysis as well as the adenohypophysis, allowing the nonapeptides to exert a wide range of peripheral effects. Of particular interest for the present review are actions that may be integrated with the central regulation of social behaviours. These include the regulation of milk ejection by OXT and the regulation of adrenocorticotropic hormone secretion by AVT/AVP (Witt, 1995; Carter, 1998; Goodson and Bass, 2001; Keverne and Curley, 2004).

Teleost fish tend to produce AVT and IT only in the POA, although other small AVT populations have been identified (Batten et al., 1990; Holmqvist and Ekstrom, 1995; Goodson and Bass, 2000b; Goodson et al., 2003). In contrast, a dramatic expansion in the number of AVT cell groups accompanied the water-to-land transition, with up to 19 AVT cell groups occurring in a single tetrapod species (the roughskin newt, *Taricha granulose*; Lowry et al., 1997). Not surprisingly, AVT exerts a diversity of behavioural actions in newts (Thompson and Moore, 2000, 2003; Rose and Moore, 2002).

Given that teleosts tend to produce AVT and IT exclusively (or near-exclusively) within the major magnocellular and parvocellular nuclei that are common to all vertebrates (Moore and Lowry, 1998; Goodson and Bass, 2001), the anatomical and behavioural "fundamentals" of nonapeptide systems are particularly tractable to examine in teleost fish. In vocalizing teleosts of the family Batrachoididae, AVT-immunoreactive (ir) and IT-ir pathways target an interconnected set of midbrain and forebrain regions that comprise an evolutionarily conserved "social behaviour network" (including amygdalar, septal, hypothalamic and tegmental regions) (Goodson and Bass, 2000b, 2002; Goodson et al., 2003). Both AVT and IT modulate fictive social vocalizations in these fish (Goodson and Bass, 2000a, b), and neurophysiologically guided tract tracings demonstrate that vocally active brain areas receive direct projections from the parvocellular POA, but not the magnocellular POA (Goodson and Bass, 2002). Given that nonapeptide distributions in

these vocal teleosts are highly similar to those in tetrapods, the tracing results suggest that the parvocellular neurons of the POA-AH give rise to the most ancient, socially relevant nonapeptide circuits in the brain. In addition to social vocalization, AVT also modulates aggression in fish (Semsar et al., 2001; Lema and Nevitt, 2004; Santangelo and Bass, 2006), and similarly, the nonapeptides influence aggression and social communication across a wide range of other vertebrate taxa (Goodson and Bass, 2001; Thompson et al., 2006).

The expansion of AVT/AVP circuitries in tetrapods includes some features that are taxonomically widespread and some that are taxon-specific. For instance, of the 19 AVT populations in newts, several appear to be highly derived, but many are likely homologous to cell groups in other tetrapod species, and thus represent the ancestral tetrapod state (Lowry et al., 1997; Moore and Lowry, 1998). Most notable in this regard are the AVT/AVP neurons of the suprachiasmatic nucleus, which are fundamental to the expression of many biological rhythms, and those of the medial bed nucleus of the stria terminalis (BSTm), which are implicated in a variety of social behaviours. The homology of AVT/AVP neurons in the BSTm is particularly well supported by the virtual ubiquity of sex-steroid sensitivity in these cells and their projections to the lateral septum (LS). Sexual dimorphisms in cell number and fibre density (male > female) are also commonly observed in the AVT/AVP circuitry of the BSTm and LS (Moore and Lowry, 1998; De Vries and Panzica, 2006).

Standing in stark contrast to the conservation described above are the distributions of the nonapeptide receptors. Whereas receptor distributions for most neurochemicals are relatively conserved, those for OXT and AVT/AVP are highly variable and species-specific, with widespread and qualitative differences being observed even between species that are very closely related (Insel et al., 1991, 1994; Goodson et al., 2006). This observation clearly accounts for much of the functional diversity and evolutionary plasticity that is observed within the vasotocin family, as described below.

Evolutionary plasticity of nonapeptide systems generates social diversity

If we examine the spectrum of neurotransmitters, neuromodulators and receptor types that are present in vertebrates, one thing becomes immediately clear — that the nonapeptides exhibit levels of plasticity, sexual dimorphism and species-specificity that are far beyond that of other neurochemical systems. Certainly there are highly conserved features, as described above (e.g. in peptide structure and major cell groups), but nonapeptide receptor distributions evolve at an astonishing rate and nonapeptide systems exhibit myriad hormone-dependent features. Thus, there is a general tendency for certain features of nonapeptide systems to be temporally plastic and evolutionarily labile. This means that nonapeptide circuits provide more "grist for the mill" of behavioural evolution than do other neurochemical systems, and we can therefore expect to find an extraordinary level of species-specificity in nonapeptide anatomy (particularly in receptor distributions) and related behavioural functions. At the same time, since the nonapeptides are arguably the "easiest" thing for natural selection to target in relation to behaviour (given the available variation and plasticity), we can expect to find a great deal of mechanistic convergence in relation to specific kinds of derived behaviours.

Sex differences comprise the most common variation in AVT/AVP systems (De Vries and Panzica, 2006), thus it is not surprising that AVT is associated with highly derived forms of sexual plasticity (Foran and Bass, 1999). Teleost fish are the most remarkable in this respect and exhibit a dazzling array of sexual plasticity, including serial sex change, terminal sex change (both male-to-female and female-to-male) and the presence of multiple reproductive phenotypes (Bass and Grober, 2001). The nonapeptides are implicated in all of these forms of plasticity. For instance, rapid increases in AVT mRNA are correlated with dominance behaviour during socially-mediated sex change in bluehead wrasse (*Thalassoma bifasciatum*) (Godwin et al., 2000), and endogenous AVT is necessary for the assumption of dominance behaviour in this species (Semsar and Godwin, 2004).

Whereas sex-changing fish may transiently express both male-typical and female-typical traits, some sexually polymorphic species stably express phenotypes that display a mixture of sex-typical characters. Polymorphic species therefore offer the opportunity to examine the extent to which sex-typical peptidergic mechanisms can be dissociated from gonadal sex. The most direct evidence of such dissociation comes from the studies of vocal-motor physiology in the sexually polymorphic plainfin midshipman fish (*Portichthys notatus*). Fictive social vocalizations in the midshipman can be electrically elicited from a number of brain areas, including the AH (Goodson and Bass, 2002), and delivery of nonapeptides and antagonists into the AH produces morph-specific effects.

The ancestral male morph in the midshipman is the Type I male, which vocally courts females and defends a nest site. AVT inhibits vocal-motor bursting in Type I males, whereas AVP V_1 antagonist facilitates bursting. Administrations of IT and an OXT antagonist are without effect. Females exhibit a pattern of nonapeptide effects that is the reverse of Type I males, such that they are sensitive to IT manipulations but not to AVT manipulations (Goodson and Bass, 2000a).

The third reproductive phenotype, the Type II male morph (which sneak or satellite spawns), is an evolutionarily derived phenotype that expresses an interesting amalgamation of sexual characteristics. Their appearance and size are similar to females; they exhibit the simple vocal repertoire of females; and like females, they visit nests of Type I males only to spawn, leaving the Type I males to provide parental care (Bass, 1996). However, relative to body size, their testes are many times larger are those of the Type I males. Hence, in Type II males, the POA-AH regulate the pituitary–gonadal axis in a hypermasculinized manner. Despite this, hypothalamic delivery of nonapeptides and antagonists in Type II males produces a pattern of effects that is virtually identical to females, not Type I males (Goodson and Bass, 2000a).

The results in midshipman demonstrate that gonadal sex and sex-typical behavioural modulation can be uncoupled from each other, even within brain regions that regulate the pituitary–gonadal axis. The developmental mechanisms that differentiate morphs remain to be determined, and it is not yet clear whether midshipman possesses sex chromosomes. However, findings in mice clearly show that both genomic and non-genomic factors can contribute to the sexual differentiation of AVP systems (De Vries et al., 2002). Such dual effects should offer natural selection greater flexibility in relation to AVT/AVP systems, and could represent a mechanism whereby sex-typical behavioural modulation can become uncoupled from gonadal sex.

Although the functional significance of morph-specific nonapeptide effects in the midshipman remains to be elucidated, it is notable that similar sex-specific effects of OXT and AVP are observed in monogamous prairie voles (*Microtus ochrogaster*): Endogenous OXT is required for pair bonding in female prairie voles, whereas endogenous AVP is required for pair bonding in males (Insel and Hulihan, 1995; Young and Wang, 2004). Whether these similarities in voles and midshipman reveal a conserved vertebrate trend or represent convergent evolution remains to be determined.

A more clear case of evolutionary convergence is exhibited between monogamous voles and other monogamous mammals. Monogamous and non-monogamous species of *Peromyscus* mice and *Microtus* voles exhibit widespread differences in the distributions of AVP V_{1a} receptors and OXT receptors (Insel et al., 1991, 1994; Insel and Shapiro, 1992). In general, though, the pattern of species differences in the vole comparisons is different than the pattern of species differences in mice, suggesting that not all of the variation in receptor distribution is related to mating systems. Even so, multiple studies of monogamous and non-monogamous species (including voles, mice and primates) point toward two brain areas as being relevant for monogamous pair bonding — the nucleus accumbens in the case of OXT receptors, and the ventral pallidum in the case of AVP V_{1a} receptors (Wang et al., 1997; Young et al., 1999; Liu and Wang, 2003; Lim et al., 2004a). Convergent evolution is particularly clear in the case of V_{1a} distributions, since in each comparison of monogamous and non-monogamous species, the monogamous species exhibits a

higher density of V_{1a} receptors in the ventral pallidum (Young and Wang, 2004). These species differences are both necessary and sufficient to account for species differences in behaviour, as established through site-specific manipulations of V_{1a} gene expression (Pitkow et al., 2001; Lim et al., 2004b; Lim and Young, 2004).

Vasotocin and the evolution of avian sociality

In addition to differing in mating system, the monogamous and non-monogamous rodents discussed above diverge in other aspects of social organization. For example, the monogamous vole species often form small groups and exhibit biparental care (Getz et al., 2005), whereas the non-monogamous species typically do not. Given that the nonapeptides are relevant for the regulation of various affiliative and paternal behaviours in addition to pair bonding (Wang et al., 1994; Parker and Lee, 2001; Bales et al., 2004), the question arises as to whether peptidergic mechanisms evolve specifically in relation to a given aspect of social organization (such as mating system, parental care, or sociality) or whether multiple dimensions of behaviour are obligatorily linked together in relation to nonapeptide function. However, rodents do not offer good opportunities to address these questions, since the various aspects of behaviour cannot be adequately uncoupled in comparative studies of different species (for discussion, see Goodson et al., 2006).

In contrast to rodents, however, some bird groups offer excellent opportunities to isolate various aspects of social organization as quasi-independent variables. Finches and waxbills of the family Estrildidae are particularly useful in this regard, since sociality (i.e. grouping behaviour) can be isolated from other aspects of behaviour and ecology. The estrildids are all monogamous (forming long-term or life-long pair bonds) and exhibit biparental care. Most of the species are moderately social — flocking during the non-breeding season and loosely distributing for breeding. However, some species have evolved to be gregarious year-round and a few species have evolved highly colonial breeding. At the other end of the continuum, a small number of estrildid species live year-round as relatively asocial male–female pairs and aggressively defend territories (Skead, 1975; Goodwin, 1982). Our studies have included five species of estrildids, including two territorial species that likely evolved territoriality independently; two highly gregarious species that independently evolved coloniality; and an intermediate, modestly gregarious species (Goodson et al., 2005, 2006; Goodson and Wang, 2006).

Our primary research focus in these birds has been on the response characteristics of the AVT neurons in the BSTm. AVT/AVP cells of the BSTm likely contribute to the regulation of multiple affiliative behaviours, including pair bonding in rodents (Young and Wang, 2004; De Vries and Panzica, 2006), but no research had previously determined how different classes of social stimuli elicit responses in these neurons. To address this issue, we used double-label immunocytochemistry to examine the induction of Fos (a marker of neuronal activity) in AVT-ir neurons following a control manipulation or exposure to a same-sex conspecific through a wire barrier (both sexes were examined, although no sex differences were observed). A significant interaction effect was obtained (species $\times$ condition), reflecting the fact that in the territorial species, exposure to a same-sex conspecific tended to *decrease* the colocalization of immunoreactive AVT and Fos, whereas colocalization tended to increase in the highly gregarious species. The modestly gregarious species exhibited virtually no change in the colocalization of AVT and Fos (Goodson and Wang, 2006). Importantly, the testing paradigm that we employed limits the overt expression of social behaviours; thus, the species differences in Fos response should primarily reflect differences in perceptual or motivational processes, not differences in behavioural response.

The data just described suggest the hypothesis that BSTm AVT neurons may exhibit increases in Fos expression following exposure to "positive" stimuli that normally elicit affiliative responses, but not to "negative" stimuli that normally elicit aggression or aversion. Such a valence sensitivity could readily account for divergent responses to same-sex stimuli in territorial and gregarious

species. Two additional findings firmly support our hypothesis. First, in the territorial violet-eared waxbill (*Uraeginthus granatina*), exposure to a same-sex conspecific produces a significant decrease in AVT-Fos colocalization, whereas exposure to a positive social stimulus, the subject's pair bond partner, produces a very robust increase (Fig. 2A). Similarly, in the highly gregarious zebra finch (*Taeniopygia guttata*), significant increases in AVT-Fos colocalization are observed in a mate competition paradigm if the subjects are allowed to court, but not if the subjects are aggressively subjugated (Fig. 2B; Goodson and Wang, 2006). Recent findings from our laboratory further demonstrate that the AVT-ir neurons of the BSTm are sensitive only to positive *social* stimuli, since positive non-social stimuli are without effect (Goodson, unpublished observations).

Consistent with these findings, we found that: (1) the two highly gregarious, colonially breeding species have significantly more AVT-ir neurons in the BSTm than do the other species, (2) the territorial species have lower baseline levels of AVT-Fos colocalization than do the gregarious species and (3) the three gregarious species have

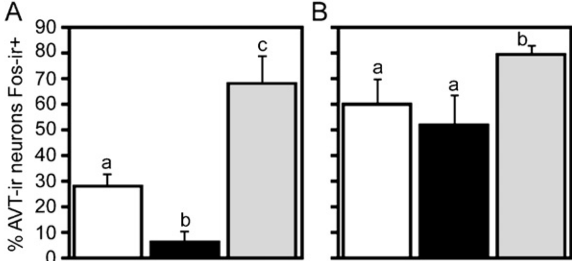

Fig. 2. AVT neuronal responses to social stimuli (adapted with permission from Goodson and Wang, 2006). (A) Percentage of AVT-ir neurons in the BSTm that express Fos-ir nuclei (means $\pm$ SEM) in the relatively asocial violet-eared waxbill following exposure to a control condition (open bars), a same-sex conspecific (black bars) or the subject's pair bond partner (grey bars). Different letters above the bars indicate significant group differences (Mann–Whitney tied $p<0.05$, following significant Kruskal–Wallis tied $p=0.003$). Total $n=16$. (B) AVT + Fos colocalization in control (open bars), subjugated (black bars) and non-subjugated (grey bars) zebra finches exposed to mate competition (Kruskal–Wallis tied $p=0.03$). No sex differences were observed and sexes are shown pooled. Total $n=15$.

significantly higher densities of V_{1a}-like binding sites in the LS than do the territorial species (Fig. 3) (Goodson and Wang, 2006; Goodson et al., 2006). In rodents, septal V_{1a} receptors promote active social interaction and facilitate social recognition (Landgraf et al., 2003) – functions that should be in higher demand for gregarious species than for territorial species.

Sociality and septal neuropeptides: what is being modulated?

Manipulations of septal AVT produce results that are consistent with the results described above, and support the idea that the AVT projection from the BSTm to the LS promotes affiliative behaviour. For instance, intraseptal AVT infusions reduce resident–intruder aggression in two species of songbirds that independently evolved territoriality — the violet-eared waxbill (Goodson, 1998b) and the field sparrow (*Spizella pusilla*; Fig. 4A) (Goodson, 1998a). In the colonial zebra finch, AVT actually increases aggression during competition to court (Goodson and Adkins-Regan, 1999; Goodson et al., 2004); however, this aggression is specifically linked to appetitive sexual behaviour (i.e. an affiliative context) (Adkins-Regan and Robinson, 1993).

These context-dependent effects on aggression are consistent with the hypothesis that AVT is influencing a broader emotional state such as anxiety. For instance, heightened anxiety may reduce aggression in a territorial bird that is faced with an intruder, but increase aggression in a gregarious zebra finch that is competing for a mate. Similar context-dependent effects are observed in male field sparrows housed outdoors on semi-natural territories. As just mentioned, resident–intruder aggression in field sparrows is decreased by intraseptal AVT infusions. However, spontaneous use of an agonistic song type during the “dawn chorus” (a time of elevated singing) is increased (Fig. 4B, C) (Goodson, 1998a). Both the decrease in overt aggression (when faced with an actual intruder) and the increase in agonistic singing (when an animal is attempting to keep

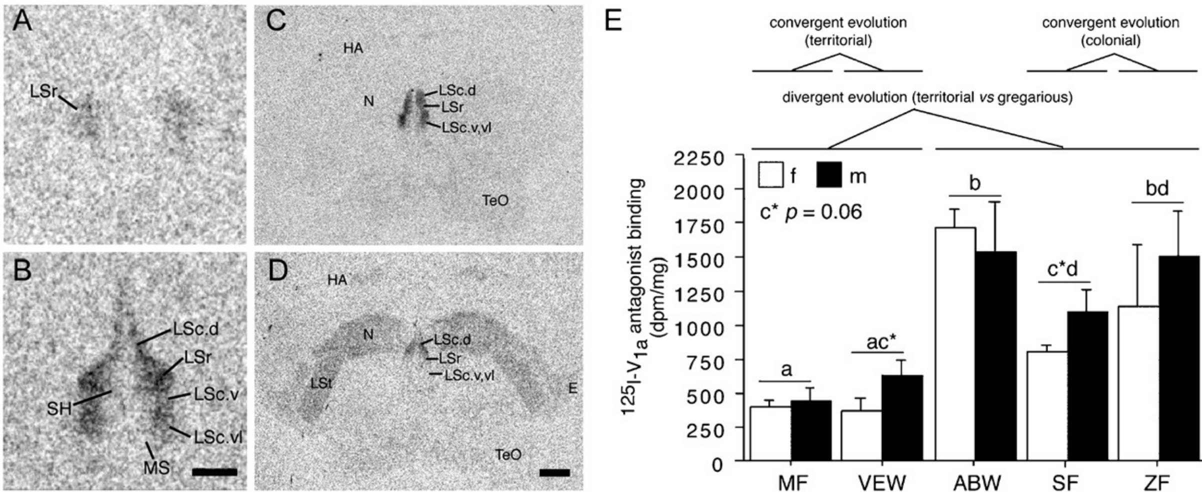

Fig. 3. Species differences in linear ^{125}I-V$_{1a}$ antagonist binding (adapted with permission from Goodson et al., 2006). (A, B) Representative binding in the septum of the territorial violet-eared waxbill (VEW; A), and moderately gregarious Angolan blue waxbill (ABW; B). (C, D) Representative sections for a male Angolan blue waxbill and male spice finch (colonial), respectively, showing species differences in binding for the nidopallium (N) and other areas of the forebrain. (E) Linear ^{125}I-V$_{1a}$ antagonist binding in the dorsal (pallial) portion of the lateral septum (LS), shown as decompositions per minute per milligram (dpm/mg; means ± SEM). Different letters above the error bars denote significant species differences (Fisher's PLSD following significant ANOVA; $p < 0.05$). The scale bar in B corresponds to 500 μm in panels A–B; the scale bar in D corresponds to 1 mm in panels C–D. Abbreviations: E, entopallium; HA, apical part of the hyperpallium; LSc, caudal division of the lateral septum (dorsal, ventrolateral and ventral zones denoted as LSc.d, LSc.vl and LSc.v, respectively); LSr, rostral division of the lateral septum; LSt, lateral striatum; MS, medial septum; N, nidopallium; SH, septohippocampal septum; TeO, optic tectum.

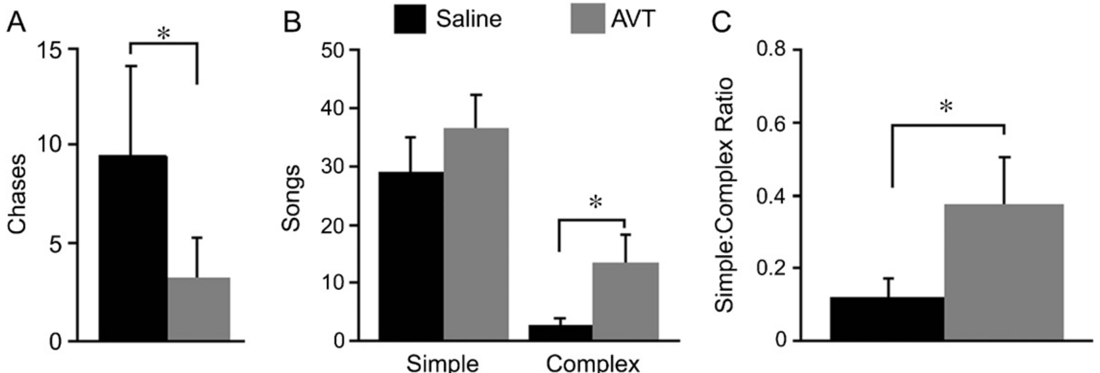

Fig. 4. Effects of intraseptal AVT infusions in male field sparrows housed on semi-natural territories (field-based flight cages placed in natural habitat; adapted with permission from Goodson, 1998a). (A) Chases during a 15-min resident–intruder test following infusion of saline control or 100 ng AVT. (B) The number of simple and complex songs given spontaneously during the dawn singing period; these song types are multipurpose and strictly agonistic, respectively (Nelson and Croner, 1991). (C) The ratio of complex to simple songs, showing an increase in the agonistic content of song following intraseptal infusion of AVT. All data are shown as means ± SEM; $^{*}p < 0.05$, Wilcoxon signed ranks; $n = 7$.

other animals away) could potentially result from an increase in anxiety.

In support of this idea, septal V_{1a} receptors influence anxiety in rodents (Landgraf et al., 1995) and V_{1a}-like receptors modulate neural responses of the LS to stress in song sparrows (*Melospiza melodia*) (Goodson and Evans, 2004). In general, manipulations of AVT/AVP that influence social behaviour also tend to influence anxiety (Pitkow et al., 2001; Bielsky et al., 2004).

Neuromodulatory patterning: overlapping circuits and distinct behavioural states

Different populations of AVT/AVP neurons can have different (even opposing) functional profiles and yet exhibit overlapping projections to the same brain areas. For instance, the AVT/AVP neurons of the BSTm appear to promote affiliative responses to social stimuli (Young and Wang, 2004; Goodson and Wang, 2006) and inhibit resident–intruder aggression (Goodson, 1998a, b), whereas AVT/AVP neurons in PVN are well-known to regulate behavioural and physiological responses to stress (Engelmann et al., 2004). Yet other AVP neurons of the AH promote offensive, resident–intruder aggression (Ferris et al., 1997; Delville et al., 2000; Gobrogge et al., 2007). These findings are hard to reconcile with each other, particularly when we consider that the projections of the different populations can be closely juxtaposed and that AVT/AVP often act in a paracrine fashion. That is, AVT/AVP signals from the diverse populations are clearly going to be mingled at their postsynaptic targets. The LS provides a concrete example: although the BSTm provides the only direct AVP innervation of the LS (De Vries and Buijs, 1983; De Vries and Panzica, 2006), neurophysiological studies indicate that AVP projections from the PVN also influence LS activity (Disturnal et al., 1986, 1987). Dendritic release of the nonapeptides ((Landgraf and Neumann, 2004) provides still another means of mingling.

How can AVT/AVP influence behaviour in a context-dependent manner if it is released in response to such disparate stimuli as sexual, stressful, or agonistic events? Or, as in the case of AVT/AVP arising from the BSTm and AH populations, have directly opposite effects on the same kind of behaviour? The answer to this paradox becomes clear if we examine the functional properties of basal forebrain ("limbic") circuits that regulate behaviour. The "social behaviour network" is characterized by extensive interconnectivity and a relative lack of linearity (Newman, 1999; Goodson, 2005). Each "node" in this network is involved in the regulation of multiple forms of social behaviour, including those that may seem to be incompatible with each other. However, it is likely not the amount of activity at any one node that determines the form of behavioural output. Rather, it is most likely the distinct *pattern* of activation across the entire network that generates distinct behavioural states. Note that although there are two different models for these circuits (Newman, 1999; Swanson, 2000; Choi et al., 2005), both require that we consider the relative amount of activity across the network nodes — that is, the overall topography of the activation pattern.

By this conceptualization, it does not matter that the PVN and BSTm may each produce the same effect on a given postsynaptic target (e.g. the LS). What is important is that AVT/AVP projections from the PVN and BSTm will each elicit a different *pattern* of postsynaptic effects across the social behaviour network, since the projections from these populations are not going to be entirely overlapping. One pattern may promote stress or aversion behaviour, and the other may promote affiliation. Hypothetically then, evolutionary modifications to behaviour will involve changing the relative strengths of functionally distinct nonapeptide systems (arising in the BSTm, PVN, AH, etc.), which would yield different patterns of postsynaptic modulation, and hence different patterns of behaviour. Such changes to weighting could be achieved by various means, including evolutionary modifications to cell number; basal and socially elicited levels of gene activity; and postsynaptic receptor densities. All of these things have occurred during social evolution in the estrildid finches and/or microtine voles, with good evidence for both evolutionary divergence and

convergence in relation to behaviour (Young and Wang, 2004; Goodson et al., 2006; Goodson and Wang, 2006). Although not yet examined in an evolutionary context, modifications to the various modes of intracerebral release and signalling could likewise produce dramatic variations in behaviour. These modes include volume transmission of neuropeptides following dendritic release, and more targeted release from axon terminals (Landgraf and Neumann, 2004). Almost limitless combinations of these modes could be employed across behaviourally relevant brain regions, yielding an endless variety of context-dependent and species-specific patterns of neuromodulation.

Summary

Nonapeptide circuits are an evolutionarily ancient component of the brain, and they exhibit numerous anatomical and functional features that are strongly conserved across the vertebrate classes. Nonetheless, certain features are evolutionarily plastic, particularly receptor distributions, which allow the nonapeptides to influence behaviour in a species-specific manner. At least in some cases, evolution in nonapeptide circuits appears to take a predictable course, given that various anatomical and functional features have evolved both divergently and convergently in relation to mating system in mammals and sociality in birds. Evolutionary modifications to nonapeptide signalling may take a variety of forms, all of which likely produce species-specific patterns of neuromodulation across brain regions. Importantly, these evolutionary modifications may adjust the relative influence of the various nonapeptide cell groups, which can be functionally distinct and even functionally opposed.

Abbreviations

AH	anterior hypothalamus
AVP	arginine vasopressin
AVT	arginine vasotocin
BSTm	medial bed nucleus of the stria terminalis
IT	isotocin
LS	lateral septum
OXT	oxytocin
POA	preoptic area
PVN	paraventricular nucleus of the hypothalamus

Acknowledgement

This research was supported by National Institutes of Health grant MH62656.

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Acknowledgements

The WCNH 2007 would like to thank the following sponsors whose financial support of the meeting was greatly appreciated:

- German Research Foundation (DFG)
- Sanofi Aventis
- Regensburger Universitätsstiftung Hans Vielberth
- Serono
- Organon
- Wyeth Research
- Ferring Pharmaceuticals
- Roche
- DRG
- VfU Verein der Freunde der Universität Regensburg
- German Society of Endocrinology (DGE)
- Novartis
- Charles River Laboratories
- Journal of Neuroendocrinology
- Behavioural Brain Research
- Stress

I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 2

Sex differences in vasopressin and oxytocin innervation of the brain

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Abstract: Sex differences in vasopressin and oxytocin expression are helpful in studying the anatomy and function of vasopressin innervation of the brain. They also provide insight in the function of neural sex differences in general. This paper will discuss nature, cause and possible significance of these sex differences, focusing on vasopressin projections from the bed nucleus of the stria terminalis and the medial amygdaloid nucleus, which show some of the most consistently found sex differences among vertebrates.

Keywords: bed nucleus of the stria terminalis; medial amygdaloid nucleus; testosterone; estradiol; hyena; prairie vole; septum; sex chromosomes; parental behaviour; social recognition memory; aggressive behaviour

Introduction

An impressive number of studies have implicated vasopressin (AVP) and oxytocin (OXT) in centrally regulated functions and behaviours. Initially, such studies focused on functions such as learning and memory (De Wied, 1969), cardiovascular functions (Versteeg et al., 1983), thermoregulation (Cooper et al., 1979; Kasting, 1989), territorial (Ferris et al., 1984) and reproductive behaviours including parental behaviour (Bohus, 1977; Pedersen et al., 1982; Södersten et al., 1985; Wang et al., 1994a). More recently, the focus has shifted to prosocial behaviours. A Medline search using 'vasopressin', 'vasotocin', 'oxytocin' and 'social behaviour' as keywords currently reveals over 500 papers, most of which published in the last decade. For example, these neuropeptides have been linked to social recognition memory and to parental, affiliative and aggressive behaviours (Carter et al., 1995; Albers and Bamshad, 1998; Engelmann et al., 2000; Young and Wang, 2004).

The neural substrate underlying vasopressin (AVP) and OXT's behavioural effects is less clear. For example, OXT is widely known to stimulate maternal behaviour (Lim and Young, 2006), but which OXT projections contribute to these effects is unclear. Knowing which cell groups synthesize and which terminals release AVP and OXT to modulate specific functions is crucial to understand how the brain uses these peptides to influence behaviour. Sex differences in AVP and OXT pathways offer a unique opportunity to address this question.

We found sex differences in AVP projections from the bed nucleus of the stria terminalis (BNST) and medial amygdaloid nucleus (MeA) by chance, while studying the development of what we thought were projections from the suprachiasmatic nucleus (SCN) to the lateral

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DOI: 10.1016/S0079-6123(08)00402-0

septum in rats. After finding a large variability among subjects, we repeated the study, now separating subjects by sex. This revealed a much higher AVP fibre density in males than in females from the second postnatal week onward (De Vries et al., 1981). Later, we and others traced the origin of these fibres to the BNST (De Vries and Buijs, 1983) and MeA (Caffé et al., 1987), where males showed about two to three times more AVP cells than females (Van Leeuwen et al., 1985; Miller et al., 1989b; Szot and Dorsa, 1993; Wang and De Vries, 1995).

Causes of sex differences in AVP projections

In mammals, differences in gonadal hormone levels are a main cause of neural sex differences (Becker et al., 2005). Early in life, gonadal hormones direct the differentiation of neural circuitry that will, in adulthood, generate male or female-typical functions and behaviours. These developmental effects are permanent and therefore called 'organizational effects'. For example, testosterone exposure during development increases the likelihood that animals show male sexual behaviour as adults. However, to do so, testosterone has to activate the neural circuitry underlying sexual behaviour in adulthood. This effect is transient and therefore called an 'activational effect'. Sex differences in AVP innervation of the brain depend on organizational as well as activational effects of gonadal hormones and, in mammals, possibly also directly on sex chromosomal complement (XX vs. XY).

Effects of circulating hormones in adulthood

In rats, circulating gonadal steroids drive AVP expression in BNST and MeA projections. Gonadectomy eliminates AVP expression whereas treatment with gonadal steroids reverses these changes (De Vries et al., 1984, 1985; Van Leeuwen et al., 1985; Miller et al., 1989a). These changes are relatively slow. In males, AVP immunoreactivity disappears from BNST and MeA projections in about 2–3 months (De Vries et al., 1984); mice show an equally slow decline (Mayes et al., 1988). AVP mRNA levels per cell, however, are significantly reduced only 1 day after castration while 1 week later only one-tenth of labelled cells remain (Miller et al., 1992). The lingering peptide levels in the terminals suggest that AVP projections maintain the capacity to influence brain functions for several weeks after castration.

Oestrogen versus androgen effects

Testosterone influences AVP production by androgen as well as oestrogen receptor-mediated mechanisms. In castrated male rats, estradiol, an oestrogenic metabolite of testosterone, partially restores AVP expression, whereas dihydrotestosterone, an androgenic metabolite of testosterone, does not by itself restore AVP expression. However, in combination with estradiol, it enhances AVP expression to control male levels (De Vries et al., 1986, 1994; Wang and De Vries, 1995). Oestrogen receptors are also important for AVP expression in mice, as null mutations in genes that code for aromatase (the enzyme that converts testosterone into estradiol) or for oestrogen receptor alpha dramatically reduce AVP expression (Plumari et al., 2002; Scordalakes and Rissman, 2004). Since virtually all AVP cells in the BNST and MeA in rats express oestrogen as well as androgen receptors (Axelson and Van Leeuwen, 1990; Zhou et al., 1994), steroids may influence AVP production by acting directly on AVP cells.

Hormones and sex differences in AVP expression

The activational effects of gonadal hormones on AVP expression suggest that differences in circulating gonadal hormones contribute to differences in BNST and MeA projections. However, such differences cannot fully explain all sex differences, because treating gonadectomized males and females with similar amounts of testosterone does not eliminate differences in AVP cell number and the density of their projections (De Vries and Al Shamma, 1990; Wang et al., 1993;

De Vries et al., 1994). Circulating hormones may, however, be the most important factor in species such as prairie voles, where differences in AVP expression are extreme (Bamshad et al., 1993, 1994; Wang et al., 1994a). These differences are reduced, but still substantial in voles that have been treated with similar levels of testosterone (Lonstein et al., 2005).

We tested whether gonadal hormones exert organizational effects on AVP expression by manipulating gonadal hormone levels during development and then clamping testosterone at the same level in adulthood. For example, we showed that males castrated at the day of birth or at postnatal day 7 had fewer AVP cells in the BNST and MeA and a lower AVP fibre density in the lateral septum than did male rats castrated at postnatal day 21 or as adults, suggesting that testicular secretions masculinize AVP projections around day 7. Treatment of neonatally gonadectomized rats with testosterone propionate at postnatal day 7, indeed, increased AVP fibre density in the lateral septum. It also fully restored the number of AVP cells in the BNST of neonatally castrated males but not females to the levels of control males (Wang et al., 1993). This discrepancy between males and females may be due to prenatal differences in gonadal hormones. Another possibility is that sex chromosomal complement cause differences independently of gonadal hormone levels.

Sex chromosomes in sex differences in AVP expression

We tested this possibility using a model system that can distinguish between differences caused by sex chromosomal complement (XX vs. XY) or different gonads (testes vs. ovaries) (De Vries et al., 2002). In this model, female mice with an XX genotype were crossed with males with an XY^{-} *Sry* genotype. The Y chromosome of XY^{-} *Sry* mice lack the *Sry* gene, which normally directs the differentiation of the primordial gonad into a testis (Koopman et al., 1991). XY^{-} *Sry* mice develop a male phenotype anyway, because they have an *Sry* transgene inserted on an autosomal chromosome. This cross, therefore, generates XX and XY^{-} mice of either sex depending on whether mice inherited the *Sry* transgene. Comparing XX and XY^{-} mice within sex (defined on the basis of gonad) showed that AVP innervation of the lateral septum was denser in XY^{-} than in XX animals irrespective of gonadal sex (De Vries et al., 2002; Gatewood et al., 2006). As these mice still showed respectable differences between gonadal males and females irrespective of sex chromosomal complement, hormones as well as sex chromosomal complement appear to control sexual differentiation of AVP expression in mice.

Cellular mechanisms underlying differentiation of AVP expression

Two fundamentally different sets of processes could cause differences in AVP cell number: processes such as cell birth, cell death, or cell migration, or processes that influence the phenotype of existing cells. Differential cell birth and migration are unlikely, because AVP cells are born at least a week before hormones trigger their sexual differentiation (Wang et al., 1993; Al-Shamma and De Vries, 1996). Differential cell death can probably be ruled out as well, because the sex difference remains intact in mice with a null mutation in the gene coding for the cell death factor, Bax (De Vries et al., 2005), even though this mutation eliminates other neural sex differences (Forger et al., 2004). This leaves differentiation of phenotype as the most likely cause. Circumstantial evidence supports this: essentially all AVP cells in the BNST co-express the neuropeptide galanin, but not all galanin cells co-express AVP (Planas et al., 1995). As the total number of galanin cells does not differ between males and females, Planas et al. (1995) proposed that, during development, higher levels of testosterone may increase the percentage of galanin cells that will co-express AVP. In support, AVP and galanin neurons in the BNST and MeA show the same unusual birth profile with both born days earlier than most surrounding cells (Han and De Vries, 1999).

The molecular mechanisms whereby testosterone directs more cells to express AVP in males

remain unknown. The permanency of these effects suggests that epigenetic mechanisms are involved. The detail in which the role of steroid metabolism, steroid receptor expression and sex chromosomal complement in sexual differentiation is known can be exploited to solve these questions, as it suggests diverse ways to manipulate sexual differentiation of AVP expression and therefore its underlying mechanisms.

The origin of sexually dimorphic AVP innervation

Steroid responsiveness has been helpful in differentiating projections from the most conspicuous sources of AVP innervation in the brain, i.e. the SCN, paraventricular nucleus (PVN), BNST and MeA (Fig. 1B) (De Vries and Miller, 1998). For example, our proposal that the BNST and MeA are the sources of sexually dimorphic AVP innervation (DeVries et al., 1985) has been widely accepted. This proposal, however, was based on a rather limited set of experiments performed primarily to locate the source of septal AVP innervation (De Vries and Buijs, 1983). In short, we made knife cuts ventral to the lateral septum to show that AVP-ir fibres enter the septum ventrorostrally. We injected retrograde tracers in the lateral septum, which labelled BNST cells, but not cells in the SCN and PVN, which at that time were considered to be the main source of AVP innervation in the brain. We lesioned the PVN bilaterally, which, just as had been shown for SCN lesions earlier (Hoorneman and Buijs, 1982), spared septal AVP innervation. We subsequently lesioned the BNST unilaterally (bilateral lesions caused high mortality). This decimated septal AVP innervation ipsilaterally. Together these data identified the BNST as the most important source of the innervation of the lateral septum (De Vries and Buijs, 1983).

Evidence for other projections from the BNST and MeA, however, is mainly circumstantial. After finding that castration eliminated AVP expression in the BNST and MeA and AVP fibre staining in all areas where unilateral lesions of the BNST had decimated AVP innervation, we proposed that the BNST and MeA project to all areas where castration eliminated AVP immunoreactivity, but not to areas where AVP immunoreactivity remained (DeVries et al., 1985) (Fig. 1). Later, Caffé et al. (1987) combined retrograde tracing with AVP immunocytochemistry to confirm that the BNST projects to the lateral septum and also to show that the MeA projects to the ventral hippocampus as well as the lateral septum. None of the other BNST and MeA projections have been

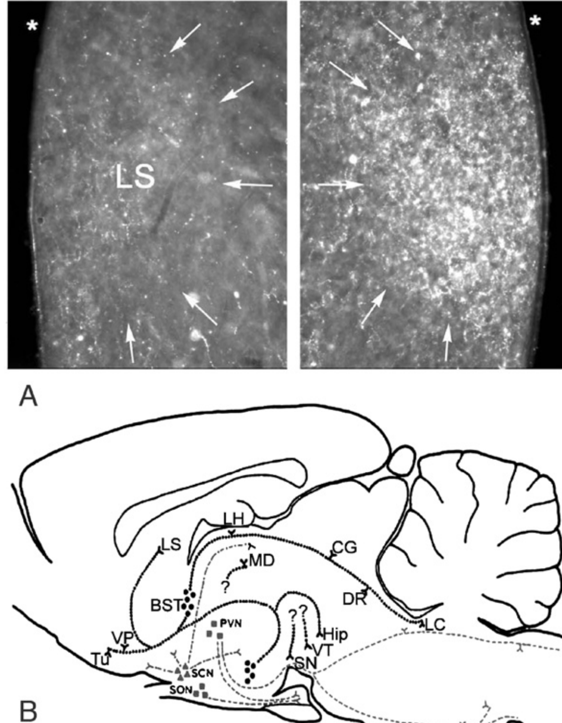

Fig. 1. Steroid-responsive AVP projections in the rat brain. (A) AVP-immunoreactive fibres (arrows) in the lateral septum (LS) of an intact female (left) and male rat (right); * lateral ventricle. (B) Diagram of most prominent AVP-ir projections in rats. Steroid-sensitive projections (black lines) run from BNST (BST; circles) and MeA (MA; circles) to the LS, ventral pallidum (VP), olfactory tubercle (Tu), lateral habenular nucleus (LH), midbrain central grey (CG), dorsal raphe nucleus (DR), locus coeruleus (LC) and ventral hippocampus (Hip). Question marks indicate projections to mediodorsal nucleus of the thalamus (MD), ventral tegmental area (VT), substantia nigra (SN), all of which disappear after castration but not after lesioning the BST. Relatively steroid-insensitive projections (grey lines) originate in SCN (triangles), PVN (squares) and supraoptic nucleus (SON, squares). Adapted with permission from De Vries and Panzica (2006).

independently confirmed. In further support, however, all presumed projections from the BNST and MeA studied so far show higher densities of AVP fibres in males than in females (De Vries and Al Shamma, 1990).

Just as much as castration effects on AVP expression inform us about the anatomy of BNST and MeA projections, they may shed light on the origin of extracellular AVP that reaches its targets via diffusion from distant release sites via a process called "volume transport" (Landgraf and Neumann, 2004). Strong proof for AVP volume transport is that push–pull cannulae or microdialysis probes have sampled AVP in areas where AVP fibres are sparse or even absent, for example, in the dorsal hippocampus (Landgraf et al., 1991) AVP in such areas may be derived from dendrites of magnocellular neurons in the PVN and SON, which can release large amounts of peptide in the extracellular space (Ludwig and Leng, 2006). In areas that receive dense AVP innervation, such as the lateral septum, extracellular AVP may be derived from local as well as distant release sites (Landgraf and Neumann, 2004). To fully understand the function of AVP signalling in these latter areas, one would like to determine the relative contributions from local and distant release sites. Gonadectomy might be helpful in this respect. For example, if gonadectomy does not reduce extracellular levels of AVP in the septum, most of it is probably derived from sources other than local BNST and MeA projections. On the other hand, a precipitous drop in AVP levels in gonadectomized rats would be consistent with BNST and MeA projections being the most significant source.

Sex differences in other AVP systems

Although PVN and SCN AVP projections do not show sex differences as global as those shown by BNST and MeA projections, partial sex differences have been found. For example, AVP innervation of the medial preoptic nucleus and periventricular nucleus of the hypothalamus likely originates in the SCN (Kriegsfeld et al., 2004). In gerbils, projections to the former nucleus are denser in males whereas the latter do not differ (Crenshaw et al., 1992). This discrepancy may be related to sex differences in the cytoarchitecture of the target, which are substantial in the medial preoptic area but insignificant in the periventricular area (Commins and Yahr, 1984). Sex differences have also been found in AVP and OXT neurons or in their vasotocin (AVT), mesotocin or isotocin counterparts in non-mammalian vertebrates in nuclei other than the BNST and MeA (Moore and Lowry, 1998; Goodson and Bass, 2001). For example, AVP neurons in the PVN are more numerous or larger in males than in females of several species, including in humans (Ishunina et al., 1999). The same is true for AVT neurons in the preoptic area in non-mammalian vertebrates, especially in fish (Goodson and Bass, 2001). Of all these sex differences, however, those in AVP projections of the BNST and MeA and in homologous projections in non-mammalian vertebrates are found most consistently, with males typically showing more cells and denser projections than females in all vertebrate classes except fish (De Vries and Panzica, 2006.)

Function of sex differences in AVP expression

The widespread occurrence of the sex differences in BNST and MeA projections suggests that they serve a function important enough to be conserved through evolution. Figuring out this function is intimately connected to determining the role of AVP in the brain. This task is made easier by the large amount of research devoted to this peptide. Ever since De Wied introduced the neuropeptide concept inspired by AVP's (and ACTH's) effects on learning and memory (De Wied, 1969), many studies have addressed AVP's effects on behaviour, some of which were mentioned at the beginning of this chapter. As these studies often involved injecting AVP or its analogues or antagonists into ventricles, it is difficult to link many of these AVP effects to any specific system. More recent studies, however, have been done with the anatomy of the central AVP pathways in mind. For example, septal AVP has been implicated in thermoregulation, osmoregulation, social recognition memory, aggressive behaviour, stress

responses and anxiety (reviewed in Dantzer and Bluthe, 1992; De Vries and Miller, 1998; Engelmann et al., 2000; Toufexis et al., 2005, 2006).

In earlier reviews of this system, I used to state that the sexually dimorphic AVP innervation was probably involved in sexually dimorphic functions or at least in functions that are strongly influenced by gonadal steroids. There are at least two problems with these statements. First, only a subset of these functions associated with septal AVP are clearly dimorphic or under gonadal steroids control, most notably, aggressive behaviour. Other functions, equally well linked to septal AVP, do not show clear sex differences. The second problem was that in making these suggestions I did not consider that the impact of the sex difference in AVP fibres might be reduced by sex differences in other systems that act in opposite directions. Here, I will discuss two extreme examples to illustrate the difficulties with assuming that sex differences in AVP expression beget sex differences in function.

Lessons from spotted hyenas and prairie voles

Spotted hyenas show unusual sexual differentiation. For example, female hyenas develop a phallus as large as that of a male (Matthews, 1939; Watson, 1877). They also show a reversal of typical sex difference in behaviour, with female hyenas being socially dominant and more aggressive than males (Matthews, 1939; Hamilton et al., 1986). Although the lack of sexual differentiation in peripheral tissue is not well understood, unusually high androstenedione levels in females during development and in adulthood may explain high levels of aggression in females (Glickman et al., 2006). As androstenedione stimulates AVP innervation in the rat brain just as well as does testosterone (Villalba et al., 1999), we hypothesized that androstenedione might also boost AVP expression in female hyenas. Indeed, we did not find the typical sex difference in septal innervation. Two of the four males studied had AVP levels similar to females whereas the two other males had almost no AVP in the septum (Rosen et al., 2006). Given that AVP has been linked to aggressive behaviour in many species (Goodson and Bass, 2001; Ferris, 2005; Goodson, this volume), this lack or perhaps even reversal of a sex difference in AVP innervation fits well with the high levels of aggression found in female spotted hyenas, just as much as the more commonly seen sex difference in AVP innervation fits with males being typically more aggressive than females. Interestingly, in Syrian hamsters, which lack AVP projections of the BNST and MeA altogether (Albers et al., 1991; Miller et al., 1999), females are as aggressive as males and tend to dominate males of similar body weight (Payne and Swanson, 1970; Huhman et al., 2003). All of these examples are compatible with the idea that sex differences in AVP innervation beget sex differences in function. They also invoke the idea that size and direction of sex differences in AVP innervation correlate directly with size and direction of sex differences in behaviour. Research in prairie voles, however, suggests that sex differences in AVP innervation cannot be that easily interpreted.

Prairie voles resemble spotted hyenas in that they are highly social (Getz et al., 1981; Carter et al., 1995). They differ in that social behaviours, including aggressive and parental behaviour, are remarkably similar between males and females (Villalba et al., 1997; Lonstein and De Vries, 1999a). Interestingly, however, the sex difference in AVP expression in prairie voles is larger than what has been reported for any other mammal (Bamshad et al., 1993, 1994; Wang et al., 1994b). In addition, reproductive status influences AVP innervation differently in males and females. In males, mating increases AVP mRNA expression in the BNST while reducing AVP-immunoreactive fibres in the lateral septum; female prairie voles do not show such changes (Bamshad et al., 1994; Wang et al.,1994b). This suggests that, in males, mating increases release of AVP from septal fibres, thereby perhaps causing the changes in social behaviour seen after mating. This appears to be true for the increase in aggression and pairbonding, which could be blocked with a V1a antagonist (Winslow et al., 1993). Mating also increases paternal responsiveness (Bamshad et al., 1994). AVP may contribute to this change as well,

as septal injections of AVP stimulate paternal responsiveness V1a antagonist injections reduce it (Wang et al., 1994a). The sex difference AVP innervation may contribute to the absence of sex differences in parental behaviour typically seen in other rodents (Lonstein and De Vries, 2000). As in other female rodents (Bridges, 1990), hormonal changes associated with pregnancy and parturition appear necessary to trigger parental behaviour in female prairie voles (Lonstein and De Vries, 1999b, Hayes and De Vries, 2007). As male prairie voles will never get pregnant let alone give birth, they need a different strategy to boost their parental responsiveness. This strategy may very well involve having a higher density of AVP innervation. In case of the prairie vole, therefore, the presence of a sex difference in AVP expression does not correlate with the size of sex differences in behaviour. The animal with the largest sex difference in AVP innervation reported to date shows the least conspicuous sex differences in social behaviour. Apparently, the sex difference in AVP expression can cause as well as prevent sex difference in social behaviour.

Dual function for sex differences in the brain

Inspired by these findings we proposed that sex differences can cause or prevent sex differences in specific behaviours or centrally regulated functions (De Vries and Boyle, 1998). This hypothesis is perfectly testable. One would predict that, in the former case, blocking AVP neurotransmission would blunt or eliminate sex differences and that, in the latter case, blocking would cause a sex difference that was not there before. In fact, such tests have already been done. For example, AVP antagonist injections block social recognition memory in male but not in female rats, thereby causing a sex difference that was previously absent (Bluthe and Dantzer, 1990). Similarly, a knockout in the V1a receptor gene reduces anxiety in male but not in female mice (Bielsky et al., 2005), exactly what one would predict if a system is more important for a function in one sex than in the other.

What does this mean for sex differences in other AVP and OXT systems? As biologists, we should consider any biological phenomenon as a possible adaptation to the circumstances. The evolutionary biologist, Dobzhanski (1973) stated that 'nothing in biology makes sense except in the light of evolution'. Roughly translated for neuroscientists this means that male neural systems have evolved to control behaviour most optimally in a male body; likewise for female systems. It would be remarkable if AVP and OXT systems would be exceptions to this rule. Therefore, the possibility that sex differences in AVP and OXT systems can cause as well as prevent sex differences in function should be considered as two obligate alternative hypotheses.

Clinical implications

A more complete understanding of the development and function of sex differences in AVP and OXT innervation may also provide novel clues as to the origin of behavioural disorders such as depression, autism and schizophrenia (De Vries, 2004; Ring, 2005; Landgraf, 2006; Carter, 2007). Each of these disorders shows sex differences in occurrence (Altemus, 2006; Goldstein, 2006; Knickmeyer and Baron-Cohen, 2006), and, in case of AVP, can be linked to variability in AVP signalling, such as elevated AVP levels in the cerebrospinal fluid or polymorphisms in the V1a receptor gene (Linkowski et al., 1984; De Bellis et al., 1993; Bartz and Hollander, 2006; Yirmiya et al., 2006). If AVP and OXT play different roles in male and female brains in humans as they do in other vertebrates (Bluthe and Dantzer, 1990; Insel and Hulihan, 1995), disruptions in receptor gene expression will affect one sex more than the other.

Abbreviations

AVP	vasopressin
AVT	vasotocin
BNST	bed nucleus of the stria terminalis
MeA	medial nucleus of the amygdala
OXT	oxytocin
SCN	suprachiasmatic nucleus

Acknowledgement

This paper was written while the author was funded by NIH grants MH47538 and MH01497.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 3

The parvocellular vasopressinergic system and responsiveness of the hypothalamic pituitary adrenal axis during chronic stress

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Abstract: Vasopressin (VP) secreted from parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) stimulates pituitary adrenocorticotropic hormone (ACTH) secretion, through interaction with receptors of the V1b subtype (V1bR) in the pituitary corticotroph, mainly by potentiating the stimulatory effects of corticotrophin-releasing hormone (CRH). Chronic stress paradigms associated with corticotroph hyperresponsiveness lead to preferential expression of hypothalamic VP over CRH and upregulation of pituitary V1bR, suggesting that VP has a primary role during adaptation of the hypothalamic pituitary adrenal (HPA) axis to long-term stimulation. However, studies using pharmacological or genetic ablation of V1bR have shown that VP is required for full ACTH responses to some stressors, but not for the sensitization of ACTH responses to a novel stress observed during chronic stress. Studies using minipump infusion of a peptide V1 antagonist in long-term adrenalectomized rats have revealed that VP mediates proliferative responses in the pituitary. Nevertheless, only a minor proportion of cells undergoing mitogenesis co-express markers for differentiated corticotrophs or precursors, suggesting that new corticotrophs are recruited from yet undifferentiated cells. The overall evidence supports a limited role of VP regulating acute ACTH responses to some acute stressors and points to cell proliferation and pituitary remodelling as alternative roles for the marked increases in parvocellular vasopressinergic activity during prolonged activation of the HPA axis.

Keywords: vasopressin; corticotrophin-releasing hormone; ACTH secretion; hypothalamic paraventricular nucleus; stress; adrenalectomy; pituitary mitogenesis

Introduction

The nonapeptide vasopressin (VP) has been long recognized as a regulator of pituitary ACTH secretion (McCann and Brobeck, 1954; Yasuda et al., 1978; Chateau et al., 1979; Baertschi et al., 1980; Buckingham, 1981). Being a weak stimulator on its own, VP can act as an important modulator of adrenocorticotropic hormone (ACTH) responses to stress by potentiating the stimulatory effect of the major regulator, corticotrophin-releasing hormone (CRH) (Buckingham, 1982; Gillies et al.,

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DOI: 10.1016/S0079-6123(08)00403-2

1982; Antoni et al., 1983). VP is produced by neurons of the hypothalamic paraventricular nuclei (PVN) and supraoptic nuclei (SON), which are organized into two major systems: (1) the magnocellular system secreting VP into the peripheral circulation from axon terminals in the neural lobe of the pituitary and (2) the parvocellular system, with axons projecting to the external zone of the median eminence from where VP is secreted into the pituitary portal circulation (Antoni, 1993). VP of magnocellular origin is responsible for water conservation in the kidney, and regulation of its secretion depends upon osmotic stimulation (Leng et al., 1999; Stricker and Sved, 2002). On the other hand, parvocellular VP expression and secretion is independent of the osmotic status and increases during stress (de Goeij et al., 1991; Antoni, 1993; Ma et al., 1997; Aguilera and Rabadan-Diehl, 2000).

The actions of VP are mediated by plasma membrane receptors belonging to the guanyl nucleotide binding protein (G-protein) family (Lolait et al., 1995a; Jard et al., 1987). The receptor present in pituitary corticotrophs is the V1b receptor subtype (V1bR), which is coupled to Gq/11 and phospholipase C, leading to activation of protein kinase C and increases in cytosolic calcium (Jard et al., 1987; Thibonnier et al., 1997). Stress, and in particular chronic stress, increases not only VP expression in parvocellular neurons but also the content of V1bR messenger ribonucleic acid (mRNA) and VP binding levels in the pituitary (Aguilera et al., 1994; Ma et al., 1997; Aguilera and Rabadan-Diehl, 2000). This contrast with the less pronounced or transient increases in CRH expression and the down-regulation of CRH receptor binding observed in repeatedly stressed rats (Aguilera, 1998). This shift in favour of a preferential activation of VP rather than CRH has lead to the proposal that VP becomes the major regulator of ACTH release during chronic stress.

This article will first present evidence supporting the prominence of the vasopressinergic system over CRH during chronic stress, and then discuss the current knowledge on the physiological importance of VP on the control of pituitary function during prolonged activation of the hypothalamic pituitary adrenal (HPA) axis.

Preferential vasopressin expression during chronic stress

Acute stress leads to rapid release of CRH and VP into the pituitary portal circulation from parvocellular neurons of the PVN (Berkenbosch et al., 1989; Plotsky, 1988; Kovacs and Sawchenko, 1996). Immunohistochemical studies have shown that in chronic somatosensory stress paradigms associated with hyperresponsiveness of the HPA axis to a novel stress, CRH stores remain unchanged but there is a progressive increase in VP stores as well as the number of CRH nerve endings containing VP (de Goeij et al., 1991). In contrast, during osmotic stimulation, a paradigm associated with an attenuated HPA axis response to stress, VP immunoreactivity in parvocellular terminals in the median eminence remains unchanged (Aguilera and Rabadan-Diehl, 2000; Grinevich et al., 2001). This correlation between CRH and VP content in parvocellular terminals and HPA axis responsiveness to stress can also be seen at the level of transcriptional regulation of the peptides.

The main determinant controlling ACTH secretion is the secretion of the stimulants CRH and VP from parvocellular terminals into the pituitary portal circulation (Antoni, 1993; Whitnall, 1993). The procedures to collect portal blood for analysis of CRH and VP present some technical challenges, especially in rodents, in which the use of anaesthetics and problems with possible contamination of portal blood with peptides in the pituitary stalk make data on the effects of stress difficult to interpret (Plotsky, 1988; Antoni et al., 1990; Tannahill et al., 1991). Studies in sheep and horse have shown rapid and equal elevations in CRH and VP in the pituitary portal circulation following stress (Engler et al., 1989; Alexander et al., 1994, 1997; Battaglia et al., 1998). The same studies in sheep show that some stressors, such as ketamine anaesthesia, cause selective increases in VP (Engler et al., 1989). However, no information is available on changes in immunoreactive peptides in the pituitary portal circulation during chronic stress. Studies in rats have shown that changes in CRH and VP transcription in paraventricular parvocellular neurons are rapid and follow closely the

activation of CRH neurons during stress (Kovacs and Sawchenko, 1996; Ma and Aguilera, 1999; Ma et al., 1999). The use of intronic in situ hybridization techniques to measure the nascent transcript or heteronuclear RNA (hnRNA) has facilitated studies on the effects of acute and chronic stress on CRH and VP transcription (Herman et al., 1991; Aguilera, 1998). These studies have revealed concomitant activation of CRH and VP transcription but with different temporal patterns; while the increases in CRH expression, assessed as changes in primary transcript or hnRNA, are transient and correlate with the stimulation of ACTH secretion, stimulation of VP expression is delayed and more prolonged (Aguilera, 1998). While in stress paradigms with sustained ACTH responses to the repeated stimulus (intraperitoneal (i.p.) hypertonic saline injection, foot shock) activation of CRH transcription is sustained during repeated stimulation, in paradigms with habituation of ACTH responses (repeated restraint, cold, repeated immune challenge) CRH transcription responses decrease progressively with repeated exposure to the homotypic stressor. As shown in Fig. 1, CRH hnRNA levels are almost undetectable in both naïve rats and in rats subjected to repeated restraint (1 h for 14 days). In naïve rats, 1 h restraint causes a marked increase in hnRNA levels but responses are absent in repeatedly restrained rats. This parallel pattern of activation of CRH transcription (and possibly secretion) and ACTH responsiveness supports a major role for CRH on acute stress responses. In contrast, VP hnRNA and mRNA responses to a repeated homotypic stress are preserved or increased, even when there is attenuation of CRH responses, such during repeated restraint (Fig. 2) (Aguilera, 1998). The mechanism of the differential transcription responses of CRH and VP within the same cell remains to be elucidated but it is likely to involve selective interaction of transcription factors with responding elements in the CRH and VP promoters.

Differential regulation of pituitary CRH and VP V1b receptors during stress

Studies in rats have shown a good correlation between changes in pituitary VP binding levels and pituitary ACTH responsiveness (Aguilera et al., 1994). For example, reduced ACTH responses during chronic osmotic stimulation are associated

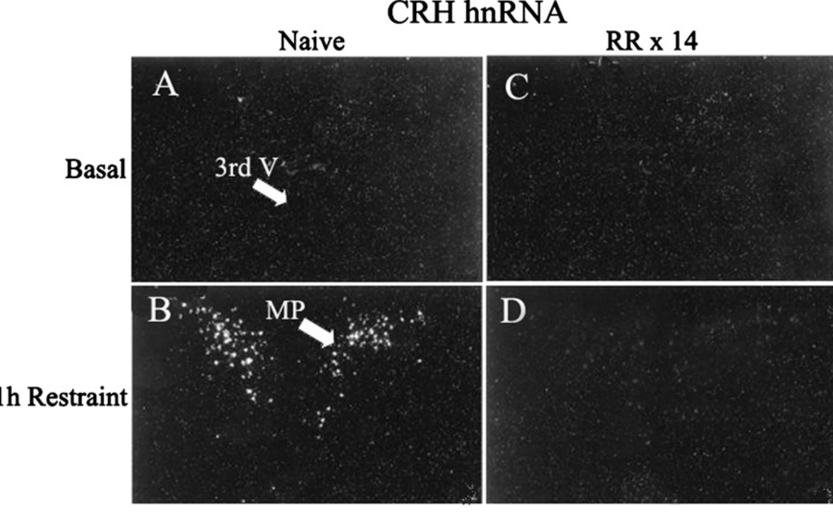

Fig. 1. Effect of acute restraint stress for 1 h on CRH hnRNA levels in the PVN of naive rats (B) or 24 h after the last restraint in rats subjected to 1-h repeated restraint for 14 days (D). Basal CRH hnRNA were almost undetectable in both naïve rats (A) and repeatedly restrained rats 24 h after the last episode (C). Darkfield photographs were taken through the medial parvocellular subdivision of the PVN (MP). 3rd V, third ventricle; RR × 14, repeated restraint, 1 h daily for 14 days.

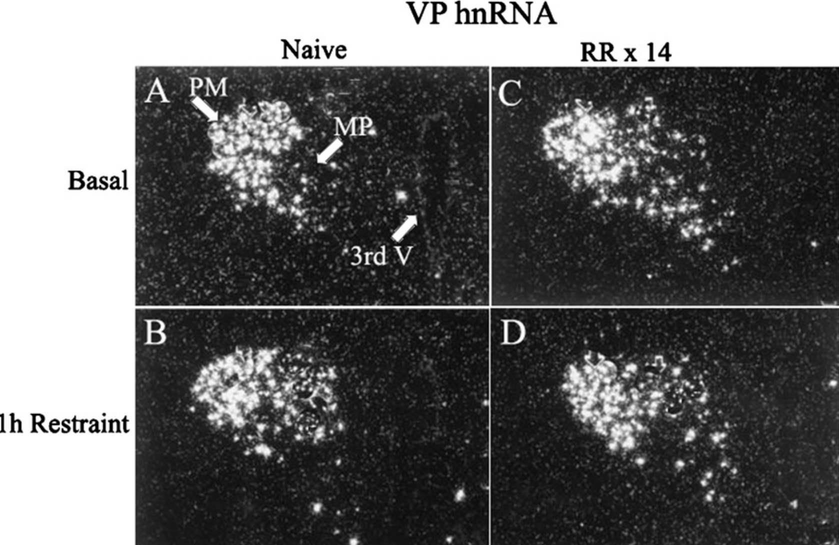

Fig. 2. Effect of acute restraint stress for 1 h on VP hnRNA levels in the PVN of naive rats (B) or 24 h after the last restraint in rats subjected to 1-h repeated restraint for 14 days (D). Basal VP hnRNA levels were undetectable in the parvocellular region of naïve rats (A), while they were markedly elevated 24 h after the last stress in repeatedly restrained rats (C). Darkfield photographs of emulsion-dipped slides were taken through the medial parvocellular subdivision of the PVN (MP). 3rd V, third ventricle; PM, posterior magnocellular; MP, medial parvocellular; RR × 14, repeated restraint, 1 h daily for 14 days.

with pituitary VP receptor down-regulation, whereas somatosensory stressors leading to ACTH hyperresponsiveness to a novel stress (repeated immobilization or repeated i.p. hypertonic saline injections, a painful stress with an osmotic component) are associated with VP receptor up-regulation (Aguilera, 1994). In general, changes in VP receptors reflect changes in the number of binding sites with no significant alteration in binding affinity (Aguilera et al., 1994).

In contrast to VP receptors, there is a poor correlation between pituitary responsiveness and the number of CRH receptors in the anterior pituitary (Aguilera, 1998). In this regard, following adrenalectomy and chronic stress, there is marked down-regulation and desensitization of pituitary CRH receptors, with decreases in both receptor number and CRH-stimulated adenylate cyclase (Hauger et al., 1988; Aguilera, 1998). It is interesting to note that VP plays an important role on the loss of CRH receptors. In the regard, CRH receptor down-regulation following adrenalectomy is markedly attenuated in the VP-deficient Brattleboro rat, and minipump infusion of VP and CRH accentuate the down-regulation induced by infusion of CRH alone (Holmes et al., 1987; Hauger and Aguilera, 1993). It is unlikely that decreases in pituitary CRH receptors during chronic stress account for the desensitization of the ACTH responses to the heterotypic stimulus, since responses to a novel stress are enhanced in these conditions (Aguilera, 1994) and CRH receptor down-regulation also occurs in paradigms associated with sustained ACTH responses to the homotypic stressor. The molecular mechanisms involved in the regulation of both CRH and V1b receptors include transcriptional, translational and post-translational events controlled by the interactive effect of glucocorticoids and CRH and VP themselves. This has been extensively reviewed elsewhere (Aguilera, 1994; Volpi et al., 2004).

The positive correlation between the content of pituitary VP receptors but not CRH receptors and pituitary ACTH responsiveness to a novel stimulus, suggests that VP receptor regulation is part of the mechanism controlling corticotroph responses and supports the concept that during chronic stress regulation of HPA axis activity switches

from CRH to VP (Ma et al., 1997; Aguilera and Rabadan-Diehl, 2000).

Does VP mediate ACTH responsiveness during chronic stress?

As discussed above, the parallel changes in VP expression in parvocellular neurons and pituitary V1bR and ACTH responsiveness during chronic stress have suggested that the increase in vasopressinergic activity is a major determinant of ACTH responsiveness to a novel stress. However, the hypothesis that VP becomes the primary regulator of ACTH responses during chronic stress has been difficult to demonstrate in studies using genetic models of VP or V1bR deficiency or pharmacological blockade of VP receptors. For example, the VP-deficient Brattleboro rat shows normal responses to most acute stressors and only a transient reduction in ACTH responses during repeated restraint (Baertschi et al., 1984; Zelena et al., 2004). On the other hand, studies in V1bR knockout mice show clear compromise of HPA axis responses to some stressors. Studies using a mouse carrying a deletion of the 3′ end of the coding region of the V1bR show reduced ACTH responses to acute hypoglycemia, lipopolysaccharide and ethanol administration but normal basal and acute restraint-stimulated ACTH (Wersinger et al., 2002; Tanoue et al., 2004; Lolait et al., 2007a, b). It is noteworthy that in contrast to rats (Aguilera, 1998; Ma et al., 1999), wild-type mice showed no habituation of ACTH responses to the repeated homotypic stress of restraint. However, V1bR knockout mice showed no response to restraint stress on day 14, suggesting that V1bR are required for sustained responses to repeated stress (Lolait et al., 2007a). Although ACTH responses are reduced, these mice are able to display sustained corticosterone responses to the repeated stimuli. Other investigators find severely deficient HPA axis responses to forced swim stress in a mouse model with a full deletion of the V1bR coding region (Tanoue et al., 2004). However, it is important to consider that the development of compensatory mechanisms due to the functional disruption of the gene since embryonic life could obscure the interpretation of the findings in models of non-inducible gene ablation.

An alternative approach is the use of VP receptor antagonists, but to date only a selective V1bR antagonist, SSR149415, is available (Serradeil-Le Gal et al., 2005). This non-peptide, orally active antagonist binds to the V1bR with nM affinity, totally blocks radiolabelled VP binding to the V1bR in transfected cells, and inhibits VP-stimulated ACTH secretion in cultured pituitary cells (Serradeil-Le Gal et al., 2002). In vivo studies have shown that SSR149415 administered intravenously, intraperitoneally or orally effectively blocked VP-induced ACTH secretion and also restraint stress-stimulated ACTH secretion (Serradeil-Le Gal et al., 2002, 2005). However, recent studies in repeatedly restrained rats show only minor effects of SSR149415 on ACTH responses to a novel stress, when given either as a single intravenous (i.v.) injection preceding a novel stress, or by repeated oral daily administration during the 14-day restraint stress (Chen et al., 2008). The same study suggests that lack of a significant effect of the selective V1b antagonist is due to a rather short biological half life of the antagonist in the experimental conditions used in the study, as shown by partial reduction of ACTH responses to exogenously injected VP (Chen et al., 2008). An additional issue to consider when interpreting the latter experiments is the fact that SSR149415 crosses the blood brain barrier and that blockade of central V1bR by the compound may influence the HPA axis responses.

In contrast, chronic osmotic minipump administration of the non-selective peptide V1 receptor antagonist, dGly[Phaa1,D-tyr(et), Lys, Arg]VP, has been shown to effectively block ACTH and corticosterone responses to exogenous VP (Subburaju and Aguilera, 2007; Chen et al., 2008), indicating reasonable blockade of V1bR in the corticotroph. In contrast to the lack of effect of the orally active V1bR antagonist, chronic administration of the non-selective V1 antagonist caused a significant reduction of ACTH responses to i.p. hypertonic saline injection, suggesting that VP contributes to the response in this acute stress paradigm (Fig. 3A). The remarkable finding in the latter study was the total inability of the

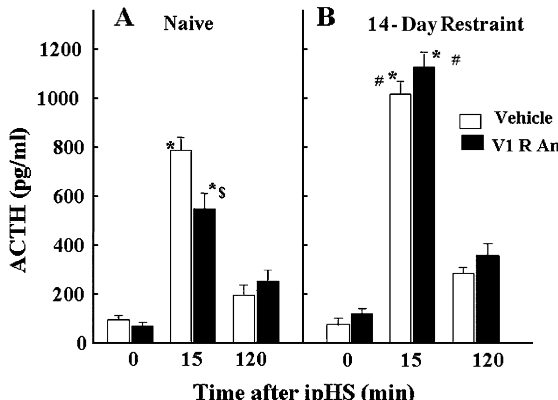

Fig. 3. The effect of chronic osmotic minipump administration of the non-selective V1 receptor antagonist, dGly[Phaa1,D-tyr(et), Lys, Arg]VP (V1R Ant), or vehicle for 14 days on plasma ACTH responses to the novel stress of i.p. hypertonic saline injection (ipHS) in conscious naïve control rats (A), or rats subjected to restraint stress for 1 h daily for 14 days (B). Data are the mean and SE of values obtained in 8 to 10 rats per group. Both groups showed a significant effect of ipHS on plasma ACTH levels ($^{*}p<0.001$). ACTH responses to ipHS in naïve rats were significantly reduced by the V1R Ant compared with the vehicle-infused controls ($^{\$}p<0.04$). ACTH responses to ipHS in repeatedly restrained rats were significantly higher than those in naïve rats ($^{\#}p<0.02$), and these enhanced responses were unaffected by the V1R Ant.

antagonist to inhibit ACTH responses to i.p. hypertonic saline injection in repeatedly stressed rats (Fig. 3B). The fact that partial (with SSR149415) or complete pituitary V1bR blockade (peptide non-selective V1 antagonist) failed to inhibit acute stress responses in repeatedly restrained rats suggest that the VP receptor up-regulation observed following repeated restraint is not required for the sensitization of ACTH responses to a novel stress.

Overall, the available evidence supports the view that VP contributes to the full ACTH response during some types of acute stress. The loss of a sustained ACTH response to repeated restraint in V1bR knockout mice suggest that VP is required for long-term HPA axis responses. However, the normal corticosterone responses indicate that ACTH secretion in these mice is sufficient to elicit full adrenocortical activation. In addition, the ineffectiveness of the peptide antagonist to modify ACTH responses during chronic stress indicates that VP does not mediate the hyperresponsiveness of ACTH responses to a novel stress and suggests alternative roles for the peptide during stress adaptation.

Alternative actions of VP in the pituitary on mitogenesis

The disproportionality between the minor effects of genetic and pharmacologic VP blockade and the prominent increase in vasopressinergic activity (manifested as an increase in parvocellular VP and pituitary V1bR expression) raises the possibility that VP not only modulates ACTH secretion but has additional functions in the pituitary. It has been shown that VP stimulates mitogenesis in a number of systems, including mouse Swiss 3T3 cells (Rozengurt et al., 1979), rat bone marrow cells following haemorrhage (Hunt et al., 1977), rat liver cells (Russell and Bucher, 1983), mesangial cell (Ghosh et al., 2001), human osteoblast-like cells (Lagumdzija et al., 2004) and the murine corticotroph tumour cell line, AtT20 (van Wijk et al., 1995). VP has also been shown to increase the number of cells incorporating deoxybromouridine (BrdU) in primary cultures of rat anterior pituitary cells (McNicol et al., 1990). Since chronic stress and adrenalectomy induce an increase in the number of corticotrophs, it is likely that VP could mediate mitogenic responses in the pituitary. This question was examined in a recent study using long-term infusion of a V1 antagonist in long-term adrenalectomized rats (Subburaju and Aguilera, 2007). As previously shown (Crane and Loomes, 1967; Rappay and Makara, 1981; Childs et al., 1989; Nolan et al., 1998), the latter study showed significant increases in the number of BrdU- and ACTH-labelled cells at 3 and 6 days, and a much larger increase at 28 days. Minipump infusion of the peptide V1 antagonist, dGly[Phaa1, D-tyr(et), Lys, Arg]VP at doses blocking the increases in ACTH and corticosterone induced by exogenous VP, for the duration of the experiment starting at the time of adrenalectomy, prevented the increases in BrdU incorporation (Fig. 4A), but not irACTH cells induced by adrenalectomy (Fig. 4B; Subburaju and Aguilera, 2007). This suggests that VP mediates mitogenic responses to adrenalectomy but that differentiation

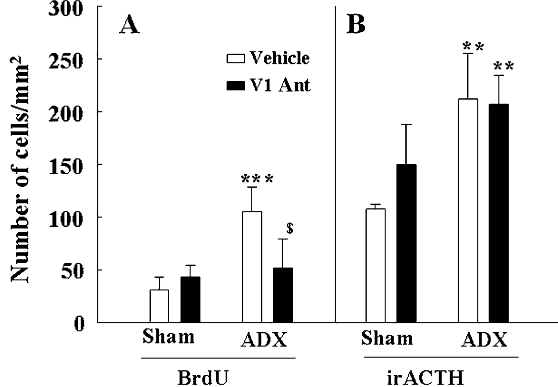

Fig. 4. The effect of chronic administration of the non-selective V1 receptor antagonist, dGly[Phaa1,D-tyr(et), Lys, Arg]VP (V1R Ant), or vehicle on adrenalectomy (ADX)-induced changes in the number of cells immunostained for ACTH or deoxybromouridine (BrdU). Rats were subjected to adrenalectomy and implantation of osmotic minipumps (Alza model 2004) containing the V1 antagonist (230 ng/h/28 days) or vehicle, and BrdU injections for 7 days before sacrifice at day 28. Rats were killed by decapitation and pituitaries fixed for BrdU and ACTH immunohistochemistry. Bars represent the mean and SE of immunopositive cells counted in pituitary sections of four rats per experimental group. $^{*}p<0.05$ vs. sham; $^{**}p<0.002$ vs. sham; $^{***}p<0.001$ vs. sham; $^{\$}p<0.05$ vs. ADX.

can occur in the absence of the peptide. However, in contrast to the findings in rats, in V1bR knockout mice, adrenalectomy for 6 or 14 days failed to increase either the number of cells incorporating BrdU or the number of irACTH cells, while inducing the expected increase in wild-type mice. This suggests that lifetime deficient pituitary vasopressinergic activity has a more profound impact on the corticotroph population. Other studies showing a reduced number of corticotrophs in the VP-deficient Brattleboro rats compared with control Long Evans rats (Tankosic et al., 1982; Schmale and Richter, 1984) also support this view.

The effects of the V1 antagonist and V1bR ablation on the number of cells undergoing proliferation during long-term adrenalectomy discussed above support the hypothesis that VP mediates the mitogenic activity in the pituitary following glucocorticoid withdrawal. In contrast, in another study, PVN lesions were unable to prevent the increase in pituitary cell proliferation induced by adrenalectomy (Nolan et al., 2004), suggesting that pituitary mitogenesis could be a direct consequence of glucocorticoid withdrawal in the pituitary. However, it is possible that VP of supraoptic origin with access to the pituitary portal circulation promotes mitogenesis (Antoni, 1993). Thus, it is conceivable that VP becomes a critical pituitary mitogenic agent during longer-term adrenalectomy.

A question still outstanding is that of the origin of the cells undergoing mitogenesis during adrenalectomy. Since adrenalectomy increases the number of corticotrophs, it would be expected that BrdU-stained nuclei co-localize with ACTH-immunoreactive cells. However, Subburaju and Aguilera (2007) found that only a minor proportion of BrdU-labelled nuclei corresponded to cells stained with ACTH or the corticotroph precursor marker, T-pit (Pulichino et al., 2004). Reports of co-localization of ACTH in pituitary cell types other than corticotrophs have suggested that mature pituitary cells can cross-differentiate (Denef et al., 2005). However, the lack of co-localization of BrdU-stained nuclei in lactotrophs, thyrotrophs, somatotrophs or gonadotrophs shown in this study is against this possibility. These observations render unlikely that the increase in corticotrophs originates from the division of existing corticotrophs or already differentiated corticotroph precursors, but suggests that recruitment of corticotrophs during adrenalectomy occurs from undifferentiated cells. Other studies have also shown lack of co-localization of BrdU in corticotrophs following adrenalectomy (Gulyas et al., 1991; Taniguchi et al., 1995; Nolan and Levy, 2001). Nolan and Levy (2006) also reported a minor incidence of mitogenesis in corticotrophs or gonadotrophs following 3- and 6-days adrenalectomy or gonadectomy in rats. In the latter study, there was no additivity of mitogenic responses to adrenalectomy and gonadectomy, supporting the view that mitogenesis in response to both stimuli occurs in an undifferentiated progenitor population (Nolan and Levy, 2006).

Of the two cell types examined as potential corticotroph progenitor cells, neither S100P-stained folliculo-stellate nor nestin-labelled stem cells have been found to co-localize BrdU. This

suggest that folliculo-stellate cells do not act as precursors for the newly-formed corticotroph following adrenalectomy, and that non-nestin expressing stem cells are probably responsible for the mitogenic responses to long-term adrenalectomy.

The fact that the increase in cells undergoing mitogenesis is VP-dependent raises the question of whether pituitary cell types other than corticotrophs express VP receptors. While the main VP receptor subtype found in the pituitary is the V1bR, the major supporting evidence for VP-mediation of mitogenic responses was obtained using an antagonist equally effective for V1aR and V1bR (Rabadan-Diehl et al., 1995). Thus, it is possible that blockade of V1aR located in pituitary cells other than corticotrophs or in the periphery could contribute to the effects of the antagonist. In addition, the published in situ hybridization image showing co-localization of V1bR mRNA and POMC mRNA (Lolait et al., 1995b), shows clusters of V1bR mRNA grains not overlaying POMC stained cells, suggesting that not only corticotrophs may express V1bR. Whether these cells correspond to pituitary progenitor cells remains to be elucidated.

Conclusions

It is clear that VP is a component of parvocellular hypothalamic-pituitary corticotroph response to stress and glucocorticoid withdrawal, and that VP stimulates ACTH secretion, mainly by potentiating the stimulatory effect of CRH. The prominence of the increases in vasopressinergic activity during chronic stress suggest that VP must have an important role in the adaptation of the HPA axis to chronic stress. Evidence from studies using genetic or pharmacological ablation of VP receptors supports the view that VP contributes to the full ACTH responses to some types of stress and sustained responses during chronic stress. However, the ineffectiveness of the peptide V1 antagonist to modify ACTH responses during chronic stress indicates that VP does not mediate the hypersensitivity of ACTH responses to a novel stress, and suggests alternative roles for the peptide during stress adaptation. Recent studies strongly suggest that one of these roles of VP is to mediate proliferative responses in the pituitary. On the other hand, the mechanisms mediating corticotroph differentiation appear to be more complex and at least in the rat are likely to involve factors other than VP. The minor co-localization of BrdU-stained nuclei in ACTH or Tpit-stained cells (or other pituitary cell types) suggests that newly produced corticotrophs following adrenalectomy and probably chronic stress originate from undifferentiated cells and not from division of existing corticotrophs. Taken as a whole, VP appears to play a minor role as a direct regulator of ACTH secretion, but one of the functions of the marked increases in parvocellular vasopressinergic activity during adrenalectomy (and probably chronic stress) is regulating cell proliferation and remodelling of the pituitary tissue.

Acknowledgement

This work was supported by the Intramural Research Program of the NIH/NICHD.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 4

Experimental approaches for the study of oxytocin and vasopressin gene expression in the central nervous system

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Abstract: Intron-specific probes measure heteronuclear RNA (hnRNA) levels and thus approximate the transcription rates of genes, in part because of the rapid turnover of this intermediate form of RNA in the cell nucleus. Previously, we used oxytocin (*Oxt*)- and vasopressin (*Avp*)- intron-specific riboprobes to measure changes in *Oxt* and *Avp* hnRNA levels in the supraoptic nucleus (SON) by quantitative in situ hybridization (ISH) after various classical physiological perturbations, including acute and chronic salt loading, and lactation. In the present experiments, we used a novel experimental model to study the neurotransmitter regulation of *Oxt* and *Avp* gene expression in the rat SON in vivo. Bilateral cannulae connected via tubing to Alzet osmotic mini-pumps were positioned over the SON. In every experiment, one SON was infused with PBS and served as the control SON in each animal, and the contralateral SON received infusions of various neurotransmitter agonists and antagonists. Using this approach, we found that *Avp* but not *Oxt* gene expression increased after acute (2–5 h) combined excitatory amino acid agonist and GABA antagonist treatment, similar to what we found after an acute hyperosmotic stimulus. Since both OXT and AVP are known to be comparably and robustly secreted in response to acute osmotic stimuli in vivo and glutamate agonists in vitro, our results indicate a dissociation between OXT secretion and *Oxt* gene transcription in vivo.

Keywords: oxytocin; vasopressin; gene expression; glutamate; heteronuclear RNA; alzet osmotic mini-pumps; osmotic regulation

Introduction

Studies of neuropeptide gene expression in the central nervous system (CNS) are usually performed by in situ hybridization (ISH) using exon-specific probes and measure the steady-state levels of mRNA, which reflect both gene transcription and mRNA degradation processes in the neuron. In contrast, measurements using intron-specific probes measure pre-mRNA or heteronuclear RNA (hnRNA) levels in the neuron which, because of the rapid turnover of the primary transcript and intermediate forms of RNA in the cell nucleus, are believed to primarily reflect the transcription rate

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DOI: 10.1016/S0079-6123(08)00404-4

of the gene. About 20 years ago, Roberts and coworkers (Fremeau et al., 1986, 1989) introduced the approach of using intron sequence-specific probes and ISH procedures to detect the pro-opiomelanocortin gene primary transcript in individual neurons of the hypothalamus. After an earlier report of using an intron-specific oligonucleotide probe for ISH (Young et al., 1986), a highly effective intron-specific vasopressin (*Avp*) riboprobe was developed (Herman et al., 1991) and widely used for studies of the regulation of *Avp* gene expression in the hypothalamo-neurohypophysial system (HNS) in vivo and in vitro. There were also efforts made to develop intron-specific riboprobes for studying oxytocin (*Oxt*) gene expression (Brooks et al., 1993). However, use of this intron 1-based probe was found to produce variable results (Rivest and Laflamme, 1995; Yue et al., 2006). Therefore, we developed and validated a new *Oxt* intron-specific riboprobe, and used this new probe, together with other well-established intron- and exon-specific *Oxt* and *Avp* probes to reevaluate *Oxt* and *Avp* gene expression in the hypothalamus under various classical physiological conditions (Yue et al., 2006). In these experiments, we found that while there was, as expected, a large increase in *Avp* hnRNA after acute salt loading, there was surprisingly no change in the *Oxt* hnRNA (Yue et al., 2006). This suggested that acute hyperosmotic stimuli produce increased *Avp* but not *Oxt* gene transcription. These observations were subsequently confirmed using quantitative real-time PCR (Yue et al., personal communication). Since both neuropeptides are robustly and equivalently secreted from the neurohypophysis following acute salt-loading (Stricker and Verbalis, 1986), it had always been assumed that the gene expression responses of the OXT and AVP magnocellular neuron (MCN) phenotypes to osmotic perturbations would also be equivalent. However, our previous studies (Yue et al., 2006) suggested that there is a significant difference in the excitation–transcription coupling mechanisms that regulate these two neuropeptide genes in the rat MCN. Given the above finding that the *Oxt* and *Avp* MCNs' gene expression responses after acute osmotic stimuli are so dramatically different, in contrast to their similar evoked secretory responses, we sought to determine whether direct application of the presumed neurotransmitter signals for secretion (Sladek, 2000, 2004; Burbach et al., 2001) onto the SONs would also produce different transcriptional responses in the OXT and AVP MCN's.

It is well established that acute or chronic increases in plasma osmotic pressure are sensed by osmoreceptors in the lamina terminalis, which in turn activate the MCNs in the HNS to secrete OXT and AVP into the systemic circulation (McKinley et al., 2004). Various lesion and inhibitor experiments have pointed to synaptic inputs to the MCNs as critical mediators of this secretory response to hyperosmotic stimuli (Sladek and Johnson, 1983; Oldfield et al., 1994; Richard and Bourque, 1995; Sladek et al., 1995; Bourque and Richard, 2001). The apparent close coupling of both acute and chronic osmotic stimuli to OXT and AVP secretion from the posterior pituitary (Stricker and Verbalis, 1986; Shoji et al., 1994; Leng et al., 2001; Ventura et al., 2005) has been attributed primarily to the action of excitatory amino acids (i.e., glutamate) and the modulation of GABA inputs on the MCNs in the HNS (Sladek and Armstrong, 1987; Richard and Bourque, 1992, 1995; Sladek et al., 1998; Swenson et al., 1998; Leng et al., 2001).

Changes in *Oxt* and *Avp* gene expression in the HNS have similarly been interpreted as being closely coupled to the same osmotically evoked synaptic inputs during chronic osmotic stimulation (Lightman and Young, 1987; Sladek, 2000; Burbach et al., 2001; Ueta et al., 2002), and in the case of *Avp* gene expression, also after acute hyperosmotic stimulation (Herman et al., 1991; Arima et al., 1999; Ueta et al., 2002). Evidence in favour of this hypothesis that synaptic input and glutamate agonists (i.e., NMDA and AMPA receptor agonists) and GABA antagonist can cause increases in *Oxt* and *Avp* mRNA in the SON comes largely from in vitro studies by Celia Sladek and her colleagues (Yagil and Sladek, 1990; Sladek et al., 1995, 1998; Swenson et al., 1998; Morsette et al., 2001).

In this paper, we further examine the hypothesis that *Oxt* and *Avp* transcription in the SON is activated by glutamate agonists (and/or GABA antagonists, e.g., bicuculline), by using a novel in

vivo experimental system and measurements of *Oxt* and *Avp* hnRNA. For this purpose, bilateral cannulae were implanted over the left and right SONs of male rats. The left SON was infused with a control solution (PBS) and its hnRNA levels were compared to those of the right SON, which received an experimental cocktail consisting of NMDA, AMPA and bicuculline in PBS delivered via ALZET osmotic mini pumps for less than 5 h. We determined the changes in *Oxt* and *Avp* gene transcription by using intron-specific riboprobes to measure *Oxt* (Yue et al., 2006) and *Avp* (Herman et al., 1991) hnRNA levels. Thus, hnRNA levels in the left (control) SONs are compared to those in the right SON in each animal, so that each experimental animal serves as its own control.

Materials and methods

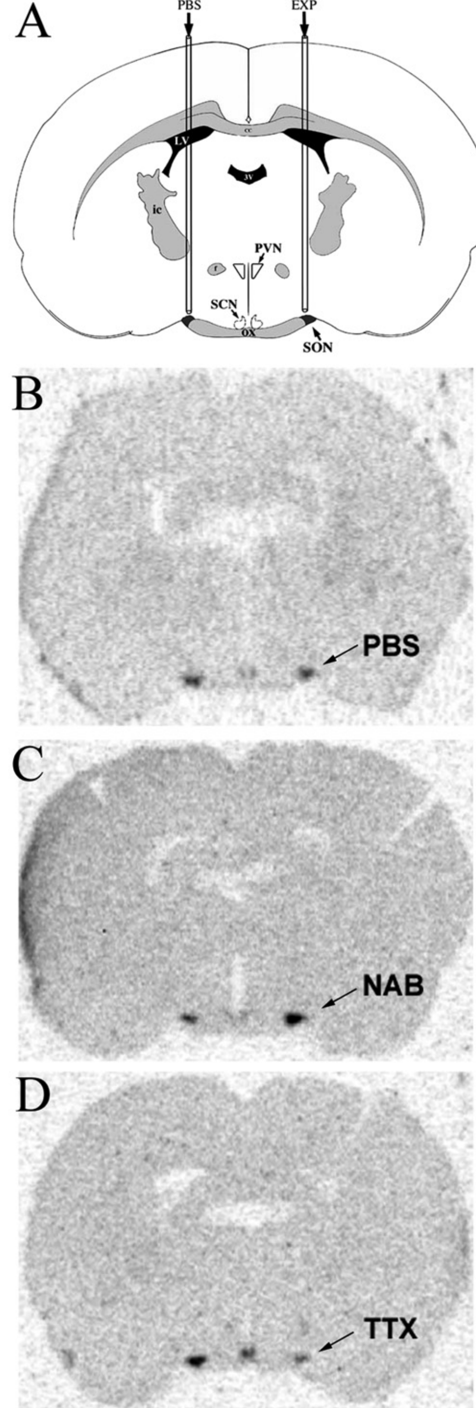
Animals

Adult male Sprague–Dawley rats (250–350 g body weight) were obtained from Taconic Farms (Germantown, NY, USA) and group housed until the time of surgery and then subsequently separated into individual cages. The animals were kept under temperature- (22 ± 1°C) and light-controlled conditions (12-h light–dark cycle, with lights on at 06:00 am). Regular rat chow and water were provided *ad libitum*. All experiments were conducted in accordance with the guidelines set forth by the NIH Animal Care and Use Committee.

Stereotaxic surgery

The surgical procedures were similar to those described elsewhere (Shahar et al., 2004). Briefly, male rats were weighed and anaesthetized with isoflurane, administered via a Stoelting gas anaesthesia adaptor for the stereotaxic instrument. Once anaesthetized, the rat was placed in the stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Using the stereotaxic coordinates from the Paxinos and Watson (1986) atlas, bilateral intracerebral cannulae connected to osmotic mini pumps were directed over the SON (see Fig. 1A). A small (1.5 mm diameter), parasagittal hole was made on each side of the skull (1.8 mm lateral to the sagittal suture and 1.30 mm caudal to the bregma) using dental burr. Two sterile stainless steel cannulae (28 gauge) 8.80 mm long, were inserted down to a level of 0.3 mm up to the dorsal border of the SON, using the following stereotaxic coordinates: 1.30 mm posterior to bregma; 1.80 mm medial lateral on each side; 8.80 mm dorsal ventral. Each cannula (Plastics One, Inc. 6591 Merriman Road, S.W. Roanoke, VA 24018) was attached via sterile PE50 tubing (Plastics One, Inc.) to a sterile model 2002, ALZET osmotic mini-pump (DURECT Corporation, Cupertino, CA, USA), and fixed to the skull with sterile acrylic dental cement (Plastics One, Inc.) bonded to sterile stainless steel screws (Plastics One, Inc.) inserted in the skull. The two ALZET osmotic mini-pumps, connected to the PE50 tubes, were placed subcutaneously on the dorsal aspect of the rat, in between the two scapulae through subcutaneous tunnels which were formed using haemostatic forceps, starting from the mid-sagittal skin incision. One pump (for the left SON) always contained the vehicle solution (sterile PBS), while the other pump (for the right SON) contained either PBS or the experimental drug. All solutions were filtered through a 0.22 μM Millipore filter. The pumps were filled at least 12 h prior to the surgery in a sterile environment and placed in the incubator (37°C) while immersed in 0.9% saline for the purpose of osmotic activation.

Following the above neurosurgical interventions, the sagittal skin incision was closed with surgical stainless steel clips and Ketoprofen was administered (5 mg/kg, diluted in 0.9% NaCl). Animals were killed within 2–5 h or 2 days of surgery. Prior to being killed, animals were anesthetized with isoflurane and immediately perfused transcardially with ice-cold 50 ml PBS followed by 250 ml of fixative solution (4% paraformaldehyde, 0.19% picric acid in 0.1 M phosphate buffer, pH 7.4). Brains were removed and post-fixed overnight in the same fixative solution (diluted 1:4 with PBS). Next, each brain was slowly agitated in a 30% sucrose solution (30% sucrose in 0.9% normal saline) for four nights changing the solution at least twice.



Experimental procedures

Control animals consisted of males that had either undergone stereotaxic surgery and infused bilaterally with PBS ($n = 2$) or males that had simply been killed and perfused without any surgical manipulation ($n = 3$). Of the males that had bilateral cannulae, one was killed 3 h after surgery, and the other was killed 2 days after surgery. The experimental male rats (NAB) received varying doses of NMDA (Sigma, St. Louis, MO, M-3262), AMPA (Sigma A1455) and bicuculline (Sigma B6889) infused over the right SON and PBS over the left SON for 2–5 h. Three animals had 100 μM NMDA and AMPA and either 50 μM, 500 μM or 2.5 mM bicuculline. Two males received a cocktail of 500 μM each of NMDA, AMPA and bicuculline (NAB). There are no significant differences in hnRNA responses between these NAB cocktails containing different doses, and these data are averaged. Three male rats received 3 μM TTX (Sigma Corp) over the right SON and PBS over the left SON for 2 days. These rats also received an osmotic stress (1.5 M NaCl i.p., 1 ml/100 kg) 2 h prior to the cardiac perfusions.

Fig. 1. Vasopressin gene expression and excitatory amino acid stimulation in the SON. (A) Schematic diagram of in vivo experimental system is illustrated. Stereotaxic surgery was performed on adult male rats and bilateral cannulae were directed over the SON. Cannulae were connected via PE50 tubing to Alzet mini-pumps containing one of three different solutions: an excitatory cocktail of NMDA, AMPA and bicuculline (NAB); TTX or PBS. In all experiments, the left SON of each male served as an internal control and received vehicle infusion (PBS), while the right SON was infused with either PBS (control solution) or an experimental solution NAB or TTX. Representative photomicrographs of *Avp* hnRNA ISH results are shown in (B–C). (B) PBS was infused over both SONs for 3 h. (C) A cocktail of NAB was infused over the right SON for 2.5 h. (D) TTX was infused over the right SON for 2 days, and 1.5 M NaCl was injected i.p. to increase *Avp* hnRNA expression acutely for 2 h. Note that the left uninhibited SON showed enhanced *Avp* hnRNA comparable to the NAB stimulated SON in (C), whereas the TTX inhibited SON (right side) did not (see text). Abbreviations: PBS, phosphate buffered saline (vehicle); EXP, experimental drug; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; OX, optic chiasm; LV, lateral ventrical; 3V, third ventrical; f, fornix; ic, internal capsule; cc, corpus callosum.

Riboprobes specific for Oxt *and* Avp

Probes used in these experiments have been previously described (Yue et al., 2006). Briefly, DNA sequences in intron 2 (In2) of the rat *Oxt* gene were used to design the PCR primers for the *Oxt* intronic probe using DNASIS-Mac v3.5 software (Hitachi Software Engineering Co., Ltd. Tokyo, Japan). The BLAST results showed that these primers are specific for the *Oxt* gene. The primers used to obtain *Oxt* hnRNA probe are: 5′-ATGAAGCTT-GTGAGCAGGAGGGGGCCTA-3′ (sense), and 5′-ATGCTGCAGCTGCAAGAGAAATGGGTC-AGT-3′ (antisense). These produced an 84 bp fragment of In2. PCR was carried out in a 50 μl reaction volume, containing 0.8 μg rat genomic DNA, 1 × PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 200 μM each of dNTPs, 200 nM each of the primers and 2.5 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). PCR was performed on a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA) and consisted of an initial 2 min, 94°C denaturation, followed by 30 cycles of denaturing (94°C, 30 s), annealing (60°C, 30 s) and extension (72°C, 45 s), followed by a final extension of 7 min at 72°C. The PCR products were loaded on the 1.5% agarose gel and purified by the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). PCR fragments were subcloned into pBlueScript II SK (+) (Stratagene, La Jolla, CA). Modified T7 (GCGCG**TAATACGACTCACTATAGGG**) and T3 (CGCGC**AATTAACCCTCACTAAAGG**) primers were used to PCR amplify the fragments and the resulting PCR products were used as templates for the synthesis of riboprobes. T7 and T3 RNA polymerases were used to obtain both sense and antisense labelled probes, respectively.

The rat *Avp* intronic riboprobe (Herman et al., 1991) was a 735 bp fragment of intron 1 of rat *Avp* gene subcloned into pGEM-3 vector (Promega, Madison, WI), and was kindly provided by Dr. Thomas Sherman (Georgetown University, Washington, DC). The latter was subcloned into pGEM-3 vectors (Mutsuga et al., 2004). Modified T7 (CATACG**ATTTAGGTGACACTATAG**) primers were used to PCR amplify fragments to make templates that were used to synthesize the riboprobes. The T7 RNA polymerase was used to synthesize the antisense of hn*Avp* riboprobe.

Labelling of the *Avp* intronic riboprobes used 40 ng of the template and 50 μCi of [α-^{35}S]-uridine 5′-triphosphate (UTP) (Perkin Elmer Life Sciences, Inc., Boston, MA), 10 mM DTT and a MAXIscript in vitro transcription kit (Ambion, Inc., Austin, TX). *Oxt* In2 labelling was performed as described above using 40 ng of the PCR product, 50 μCi of [α-^{35}S]-UTP and 50 μCi of [α-^{35}S]-CTP.

In situ hybridization for the detection of Avp *and* Oxt *heteronuclear RNA*

The ISH protocol used in this study has been previously published (Young et al., 1986; Mutsuga et al., 2004). Briefly, serial 10 μm brain sections were cut on a cryostat and placed onto poly-l-lysine coated slides (Fisher Scientific Company, Newark, DE), dried on a slide warmer for 10–30 min at 37 °C, and then stored at −80 °C. Before hybridization with riboprobes, the sections were rinsed once and washed twice for 5 min in PBS, put into 0.1 M triethanolamine-HCl (pH 8.0) containing 0.25% acetic anhydride for 10 min at room temperature, rinsed with 2 × SSC buffer and transferred through graded ethanols (75–100%) and then air-dried. Hybridization was carried out in 80 μl of hybridization solution (20 mM Tris-Cl pH 7.4, 1 mM EDTA pH 8.0, 300 mM NaCl, 50% formamide, 10% dextran sulphate, 1 × Denhardt's solution, 100 μg/ml salmon sperm DNA, 250 μg/ml yeast total RNA, 250 μg/ml yeast tRNA, 0.0625% SDS, 0.0625% sodium thiosulphate) containing 10^6 cpm denatured S^{35}-labelled riboprobe. After overnight hybridization at 55°C, the sections were washed 4 times in 4 × SSC, incubated with TNE buffer [10 mM Tris-Cl pH 8.0, 0.5 M NaCl, 0.25 mM EDTA pH8.0] containing 20 μg/ml ribonuclease A for 30 min at 37°C, and then washed twice in 2 × SSC, once in 1 × and 0.5 × SSC at room temperature, and twice in 0.1 × SSC at 65°C. The sections were rinsed in graded ethanol solutions and then air-dried. Finally, the sections were apposed to a low-energy storage phosphor screen (Amersham Biosciences, Piscataway, NJ) for 7–20 days, and developed using a phosphor imager (Storm 860, Amersham Biosciences).

Quantitative analysis of ISH

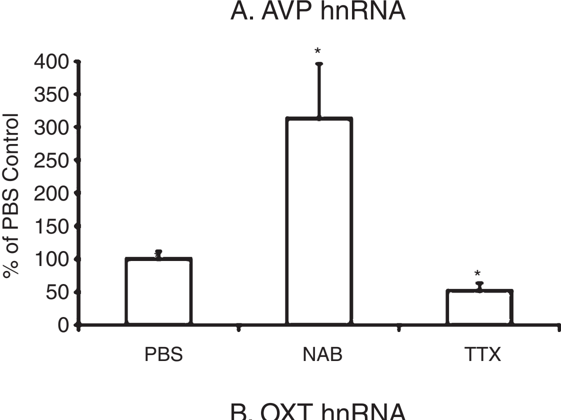
To evaluate the levels of hnRNA in the SONs, the average densities and unit areas from two representative sections in the central region of the nuclei recorded on the phosphor imager were measured using the Image Quant software version 5.2 (Amersham Biosciences).

Statistical analyses

The ratios of Avp and Oxt hnRNA of the right SONs, divided by the left SONs were statistically analyzed. Statistical significance of differences between groups was calculated by an unpaired *t*-test followed by Fisher's protected least significant differences (PLSD) post hoc test using the Statview 5.0 (SAS Institute, Inc., Cary, NC) program. Differences between groups were considered statistically significant when *p*-values were less than 0.05. Results are expressed as percent control (mean $\pm$ SEM).

Results

The experimental paradigm used in these experiments is depicted in Fig. 1A, in which ALZET osmotic mini-pumps attached to pre-positioned cannulae located over each SON in male rats were used to infuse control (PBS only) solutions over the left SON and either control or experimental solutions over the right SON. In this way, each animal's left SON serves as a control for the experimental right SON, and the hnRNA measurements on the experimental side are expressed as a percentage of the values measured on the control side. Representative ISH results for various treatments are shown in Figs. 1B, C. Figure 1B illustrates the control experiments, where both SONs are treated equally. In the experiment shown in Fig. 1B the same PBS control solution was infused over each SON for 3 h, and ISH for *Avp* hnRNA was conducted. As can be seen in Fig. 1B, there is little difference in *Avp* hnRNA levels between the two sides of the SON. The quantitative data is shown in Fig. 2. For the *Avp* hnRNA, the average ratio of the right SON over the left SON in



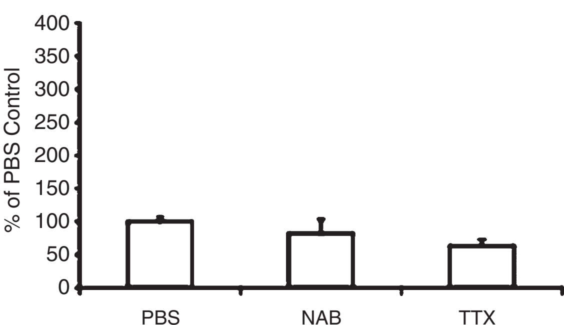


Fig. 2. Quantitative determinations of vasopressin (A) and oxytocin (B) hnRNA levels in control (unstimulated) SONs and SONs stimulated by infusions of the excitatory amino acid cocktail (NAB). The data are expressed as percentage changes in the NAB-treated (right side) SON as compared to the PBS-control (left) SON (see Fig. 1C). The control bars in the graph represent the mean control data from five animals in which both sides of the SON were equally treated (e.g., by PBS infusion only, Fig. 1B). The bars labelled NAB are from animals in which the right SON infused with PBS ($n = 5$). *Equals significant difference ($p < 0.05$) in hnRNA level between PBS and NAB stimulated SONs.

the five control animals is 1.10 ± 0.14, and is expressed as a percentage of the PBS control (left side) in Fig. 2A, indicating that there was no intrinsic difference between the left versus the right SON in the absence of drug treatment. A similar result was obtained for the *Oxt* hnRNA measurements in these control experiments, which are illustrated in Fig. 2B (PBS), where the ratio between the two sides was 1.19 ± 0.08.

Figure 1C illustrates an experiment in which the excitatory amino acid and GABA antagonist

cocktail of NAB was infused over the right SON for 2.5 h. There was an increase of *Avp* hnRNA in the NAB-treated SON (right SON) as compared to the PBS-control infused SON, indicating that the NAB was a very effective stimulus to increase *Avp* hnRNA expression in the stimulated SON. This increase was comparable to the increase in *Avp* hnRNA that we observed in the SON 2 h after an i.p. injection of 1.5 M NaCl was given to the rat (illustrated in the left side of SON in Fig. 1D, see also Yue et al., 2006). That this increase in *Avp* hnRNA in response to hyperosmotic stimulation is likely to be due to the osmotically evoked neural input to the SON, is supported by the observation that this increase is blocked by the infusion of 3 μM TTX over the right SON (Fig. 1D, right SON).

Quantitive analyses of the effects of NAB infusion over the right SON on *Avp* and *Oxt* hnRNA levels is shown in Fig. 2A, B, respectively. The average ratio of *Avp* hnRNA levels of the NAB-stimulated SON over the PBS-control SON is 3.13 ± 0.84 ($n = 5$), which represents a threefold increase in *Avp* hnRNA in the NAB-treated SON over the control SON (Fig. 2A, $p < 0.04$). In contrast, however, there was no change in *Oxt* hnRNA in the NAB-treated SONs as compared to the PBS-control SON, where the ratio between the control and treated SONs was 0.99 ± 0.19 ($n = 5$). This did not significantly differ from the control animals (Fig. 2B, $p = 0.42$). Thus, these data are consistent with the view that the *Avp* hnRNA is regulated by excitatory amino acid input and GABA modulation, but that under the same conditions *Oxt* hnRNA is not.

Discussion

A substantial literature exists that links hyperosmotically evoked excitation in the HNS with the regulated secretion of OXT and AVP into the general circulation. Studies conducted by Leng et al. (2001) show that intravenous infusions of hypertonic saline solution resulted in a linear increase in activity of both AVP and OXT cells. These authors generated a computational model which predicts that both glutamate and GABA are simultaneously released onto the SON to mediate this linear increase in activity. They tested this hypothesis in part, by administering bicuculline to the SON during an intravenous hyperosmotic infusion. Under these conditions, there was a robust increase in the slope of OXT neural activity. These data suggest that both glutamatergic and GABAergic inputs are both activated under osmotic stress, and that GABA is modulating the glutamate excitatory input which induces MCNs to release OXT and AVP into the bloodstream. Other studies have shown in vivo and in vitro that glutamate agonists and/or GABA antagonists produce increased secretion of OXT and AVP from the posterior pituitary (see Sladek, 2004, for a review). Analogous to this excitation–secretion coupling scenario, a similar view exists for excitation–transcription coupling in the HNS (Sladek, 2000; Burbach et al., 2001).

Studies in vitro using a SON–pituitary explant system have shown that increases in *Avp* mRNA and AVP secretion are tightly correlated (Yagil and Sladek, 1990). In this system, endogenous GABA tonically inhibits AVP secretion, and addition of GABA antagonists stimulated release in a dose-dependent fashion (Sladek and Armstrong, 1987). Kynurenic acid, a general excitatory amino acid antagonist, blocked increases in *Avp* mRNA and AVP secretion in response to osmotic stress (Sladek et al., 1995), and subsequent experiments suggest that both AMPA receptors (Sladek et al., 1998) and NMDA receptors (Swenson et al., 1998) mediate the AVP release. These data suggest that during osmotic stimulation, the actions of the neurotransmitters glutamate and GABA are causally involved in the increase in *Oxt* and *Avp* gene expression in the SON.

To test this hypothesis in vivo, we adapted our previously described approach of bilateral infusion of experimental solutions over the SON using ALZET osmotic mini-pumps (Shahar et al., 2004). In the latter study, we showed that osmotically evoked c-fos expression in the MCNs in the experimental SON was completely inhibited by unilateral delivery of TTX, whereas the contralateral SON that was infused by only control PBS solution underwent the expected increase in c-fos expression (see Fig. 2, in Shahar et al., 2004). In this regard, it is interesting to note that osmotically stimulated OXT

and AVP release is also blocked by application of TTX to the HNS (Ludwig et al., 1995) and that the expected increased in *Avp* hnRNA under these conditions is also inhibited (see Fig. 1D in this paper). Using this bilateral infusion approach (see Fig. 1A), we set out to determine whether direct application of glutamate agonists and GABA antagonists to the SON could evoke increases in *Oxt* and *Avp* hnRNA, as measured by *Oxt*- and *Avp*-intron-specific riboprobes.

The results of these experiments, show that *Avp* hnRNA increased after <5 h infusions of a drug cocktail comprised of a mixture of NMDA, AMPA and bicuculline (see NAB in Figs. 1C and 2A), and this increase is comparable to that found with an acute osmotic stimulus (see left SON in Fig. 1D, and Yue et al., 2006). We found in pilot experiments that applications of AMPA, NMDA or bicuculline individually are not very effective in increasing *Avp* hnRNA (data not shown), in comparison to the NAB cocktail result shown in Figs. 1C and 2A. These data for *Avp* transcription are consistent with the hypothesis of closely linked excitation–transcription coupling. In contrast, however, are the data for *Oxt* transcription (Fig. 2B) in the SON, which exhibits no change in *Oxt* hnRNA under the same conditions as when *Avp* hnRNA increases robustly. The latter data do not support the hypothesis with respect to *Oxt* gene transcription, and indicates that *Oxt* transcription does not change in response to acute osmotic stimulation, in contrast to *Avp* gene transcription which does (Yue et al., 2006). Since it is known that a chronic osmotic stimulus activates transcription from both the *Oxt* and *Avp* genes, these data suggest a profound difference in the signal-transduction mechanisms of excitation–transcription coupling must exist between the *Oxt* and *Avp* genes in the SON during acute osmotic or neurotransmitter stimulation.

Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methylisoxasole-4-propionic acid
AVP	vasopressin
CNS	central nervous system
CON	control
GABA	gamma-aminobutyric acid
hnRNA	heteronuclear RNA
HNS	hypothalamo-neurohypophysial system
ISH	in situ hybridization
MCN	magnocellular neuron
NAB	NMDA, AMPA and bicuculline
NMDA	*N*-methyl-d-aspartic acid
OXT	oxytocin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SON	supraoptic nucleus
TTX	tetrodotoxin

Acknowledgements

We thank Dr. Tal Shahar for his help in establishing the surgical technique in our laboratory, and Dr. Noriko Mutsuga for her technical assistance in ISH. This research was supported by the Intramural Research Program of the NIH, NINDS.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 5

Oxytocin knockout mice: a model for studying stress-related and ingestive behaviours

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Abstract: Oxytocin (OXT) that is released centrally is believed to be anxiolytic and have stress-attenuating effects. Oxytocin knockout (OXTKO) mice, a genetic model of OXT deficiency, have heightened corticosterone release after acute stress and greater anxiety-related behaviour in an elevated plus maze compared to wild-type (WT) mice. In the present set of experiments, we recorded the rise in body temperature, referred to as stress-induced hyperthermia (SIH), following transfer to a metabolic cage, which triggers both anxiety and corticosterone release in mice. SIH is a marker of activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system. Because corticosterone release after acute stress is typically greater in OXTKO than in WT mice, we measured SIH as a surrogate marker of corticosterone release. Following transfer to a metabolic cage, both OXTKO and WT mice increased body temperature, but to the same degree. Pregnant mice, which are known to have blunted corticosterone release to acute stress, had attenuated SIH after transfer to a metabolic cage compared to cycling mice, but both genotypes manifested the same degree of attenuation. In addition, we tested the effects of the cannabinoid receptor 1 (CBR1) antagonist/inverse agonist (AM251) upon feeding and SIH in OXTKO versus WT mice. CBR1 antagonists are known to diminish food intake and to enhance corticosterone both basally and following acute stress. Although AM251 blunted food intake, the effect was equivalent in both genotypes. The agent did not affect the SIH response compared to mice treated with vehicle. SIH is excellent for defining anxiolytic or blunted corticosterone responses (such as the stress hyporesponsiveness of pregnancy), but is limited in its ability to detect the heightened corticosterone responses that have been reported in OXTKO mice following exposure to psychogenic stress.

Keywords: stress; temperature; cannabinoids; pregnancy; mice; ingestion

Introduction

Oxytocin (OXT) is among the many centrally released neuropeptides that modulate stress and anxiety responses in mammals. OXT-signalling pathways have been proposed to attenuate stress and anxiety in a wide variety of species, including rodents (Windle et al., 1997, 2004; Neumann et al., 1998, 2000; Nishioka et al., 1998; Wotjak et al., 1998, 2001; Ebner et al., 2000; Neumann, 2002; Wigger and Neumann, 2002; Nomura et al., 2003; Huber et al., 2005; Terenzi and Ingram, 2005). Treatment of rats with exogenous OXT attenuates anxiety and stress responses (Windle et al., 1997),

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DOI: 10.1016/S0079-6123(08)00405-6

whereas treatment with an OXT receptor antagonist heightens stress and anxiety (Neumann et al., 2000; Neumann, 2002). The anxiolytic actions of OXT are most apparent in an oestrogen-rich milieu (McCarthy, 1995; McCarthy et al., 1996). Female mice that are genetically engineered to lack the gene for oxytocin (OXTKO mice) exhibit more anxiety-related behaviour in an elevated plus maze (EPM) test (Mantella et al., 2003a) and a heightened corticosterone response following exposure to an anticipatory stress such as platform shaker (Amico et al., 2004; Mantella et al., 2004), compared to female wild-type (WT) mice. Male OXTKO mice released more corticosterone than WT mice following a prolonged physical stress such as overnight food or water deprivation (Mantella et al., 2005), and both male OXTKO and OXT receptor knockout mice (Takayanagi et al., 2005) have heightened aggression compared to WT mice. Consequently OXT released within the brain is believed to attenuate stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis and to act as an endogenous anxiolytic.

When mammals are confronted with a stress or anxiety-provoking situation, sensory systems are activated providing input to limbic system components, which in turn trigger an increase in the hormonal activity of the HPA axis and sympathetic nervous system (SNS, both noradrenergic nerves and the adrenal medulla). Release of corticotropin-releasing hormone (CRH), adrenocorticotropin hormone (ACTH) and corticosterone indicates HPA activation, whereas release of epinephrine and norepinephrine reflects heightened SNS activity. Physiological markers that accompany HPA and SNS activation include increases in heart rate, blood pressure and core body temperature (Bouwknecht et al., 2007). The well-documented rise in core body temperature following an anticipatory stress or anxiety-provoking situation is termed stress-induced hyperthermia (SIH). The magnitude of the temperature rise (calculated as the difference between a basal measurement, T1, and the measurement recorded 10 min after a stress, T2, provides a quantitative index of the degree of stress or anxiety experienced by the test subject (Borsini et al., 1989; van der Heyden et al., 1997), and has been used in lieu of or in addition to hormonal markers of stress. Two versions of the test that utilize either group (Borsini et al., 1989) or single housing (van der Heyden et al., 1997) have been described in mice, but each provides comparable results. The single-housing version minimizes the number of experimental animals, and is often preferred. SIH has been used to determine if phenotypic differences in stress or anxiety exist in mutant versus WT mice; or to screen the effects of pharmacological agents upon responses to stress or anxiety. Bouwknecht et al. (2007) recently reviewed this topic.

In the present set of experiments, we compared core body temperature changes in OXTKO versus WT mice that were exposed to a stressful experience comprised of removal of an animal from its home cage, insertion of a rectal probe to record body temperature and transfer to a metabolic cage (novel environment). The process of inserting a rectal probe into an animal elevates body temperature (Borsini et al., 1989; van der Heyden et al., 1997) and circulating corticosterone concentrations in both the group- (Groenink et al., 1994) and single-housed versions of this test (Veening et al., 2004). Handling and exposure to a novel environment also raise core body temperature and corticosterone concentrations in mice (Veening et al., 2004; Bouwknecht et al., 2007). SIH is easily and rapidly performed, causes minimal discomfort and is more feasible than repeated blood sampling to measure multiple hormones in small species such as mice. Sacrifice of animals is minimized and procedures and costs are streamlined.

We studied the following groups of mice to better understand the potential role of oxytocinergic systems in modulating the SIH response: male mice, female (cycling) mice, primiparous pregnant mice in late gestation and male mice pre-treated with a selective cannabinoid 1 receptor (CB1R) antagonist/inverse agonist, AM251 (Tocris, Ellisville, MO). The experiments were designed to determine whether the presence or absence of functional OXT neuronal systems would affect the magnitude of the SIH based on prior studies implicating OXT as a modulator of the overall responsiveness of the HPA axis and SNS.

Because gender differences in the stress responses of OXTKO mice have been observed

previously (Mantella et al., 2004, 2005), male and female mice were included in the present experiments. Additionally, it has been demonstrated that the corticosterone component of stress is diminished during late pregnancy (Neumann et al., 1998, 2003; Brunton et al., 2000; Johnstone et al., 2000) but there is no information available whether SIH is also influenced. Therefore, normally cycling female animals were contrasted with pregnant females. Finally, there is evidence that oxytocinergic neuronal systems interact with endogenous cannabinoid systems (Di et al., 2003; Verty et al., 2004; Oliet et al., 2007); therefore we included an experiment to assess the magnitude of the SIH response in the presence and absence of the endocannabinoid system utilizing the CB1R antagonist/inverse agonist AM251.

Methods

Subjects

OXTKO mice used in these studies were derived from the line developed by Young et al. (1996). The OXT DNA construct of interest was introduced into stem cells from a 129 strain of mice; and the stem cells incorporating the construct were introduced into blastocysts from a C57BL/6 strain of mice (Young et al., 1996). Jackson laboratories (Bar Harbor, ME) provided the founder mice to establish the colony in Pittsburgh, which is now in the 12th generation. Mice used in the present study were genotyped as previously described (Young et al., 1996; Mantella et al., 2003a, 2004) and were from the F11 and F12 generations. WT mice from the F11 generation were the progeny of WT female and WT male matings and OXTKO mice were the progeny of heterozygote (HZ) female and OXTKO male matings. WT and OXTKO mice from the F12 generation were the progeny of HZ male and HZ female matings. Mice were studied in groups of 6–8 per genotype and were 25–35 g body weight at the time of study.

Measurement of core body temperature

Mice in each experiment were single-housed for at least 7 days prior to the start of a study or in the case of pregnant mice from early gestation. Methods for measuring core body temperature in single-housed mice before and after an acute stress have been published previously and applied in these experiments (van der Heyden et al., 1997; Bouwknecht et al., 2007). A lubricated thermocouple temperature probe (Omega, Stanford, CT) was inserted to a uniform depth of 2.0 cm into the rectum of the mouse and maintained in place for 10–15 s. A temperature reading was recorded to the nearest 0.1°. Between 1000 and 1200 h, rectal temperature was recorded in mice of each genotype before and 10 min after transfer to a metabolic cage. In pilot studies (data not shown), we determined that peak temperature is achieved 10 min after an acute stress and remains stable for 30 min before declining, in agreement with others (Bouwknecht et al., 2007).

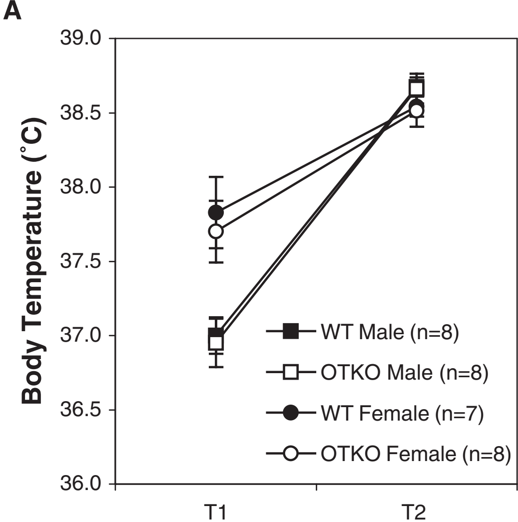
Statistical analysis

Results are presented as group means ± SEM. An effect was considered significant when $p<0.05$. Temperatures (Experiments: "Stress-induced hyperthermia in male and female mice exposed to a novel environment", "Stress-induced hyperthermia in pregnant mice exposed to a novel environment", "Effect of CB1R antagonist upon stress-induced hyperthermia in male mice exposed to a novel environment") and food intake (Experiment: "Effect of CB1R antagonist on ingestion") were analysed by two-way repeated measures analysis of variance (ANOVA). When F ratios indicated a significant effect of the factors of genotype and time, Bonferroni t tests were used for post hoc multiple comparisons between groups.

Experiments

Stress-induced hyperthermia in male and female mice exposed to a novel environment

In this experiment we measured the change in core body temperature in F12 male and female mice of both genotypes after handling and transfer to a metabolic cage. The basal temperature before stress (T1) and the temperature 10 min after the



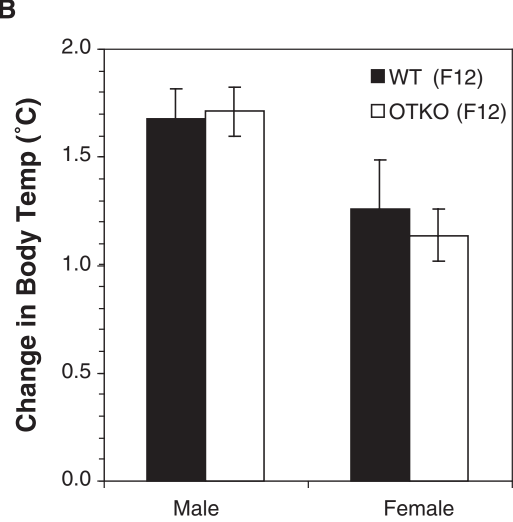


Fig. 1. (Panel A) Comparison of body temperature before, T1, and after, T2, stress in male and female wild-type (WT) and oxytocin knockout (OTKO) mice. T1 temperatures were significantly lower in male than in female mice ($p<0.05$). (Panel B) There were no genotypic differences in the stress-induced change in body temperature (T2−T1). However, because of the significant difference in the baseline, T1, temperatures in male and female mice the change in temperature was significantly greater in male than female mice ($p<0.05$).

start of the stress (T2) did not differ between OXTKO and WT mice (Fig. 1A). The change in temperature related to this stress (T2−T1) was not different between genotypes (Fig. 1B).

The basal temperature of male mice in this study was lower than that of female mice (T1, Fig. 1A), $p<0.05$, but the temperature 10 min after the start of the stress did not differ between male and female mice (T2, Fig. 1A). The change in temperature related to the stress was greater in male than female mice (T2−T1, Fig. 1B) because of the differences between genders in basal temperature.

Stress-induced hyperthermia in pregnant mice exposed to a novel environment

When late-pregnant rats or mice are confronted with a stress, the response of the HPA axis is dampened compared to non-pregnant animals and is termed stress hyporesponsiveness (Neumann et al., 1998, 2003; Brunton et al., 2000; Johnstone et al., 2000). Using hormonal correlates of HPA activation, Douglas et al. (2003) reported diminished ACTH and corticosterone responses following exposure to either forced swimming or novel environment (e.g. 10 min confinement in a glass jar). As noted in that study, the high oestrogen environment of late pregnancy induces a physiological elevation in corticosterone-binding globulin (CBG), which binds a large fraction of the circulating corticosterone. The changes in CBG result in high basal and stimulated total corticosterone concentrations when compared to non-pregnant mice. Importantly, free corticosterone, the biologically active form of the hormone, is not altered. As a result of the changes in CBG, measurements of corticosterone in late-pregnant mice are difficult to interpret without a concurrent measure of either CBG or free corticosterone. However, commercially available assay kits for corticosterone measure total, not free, corticosterone. A method for measurement of free corticosterone in mouse plasma is not routinely available and measurement of CBG requires that additional blood be drawn for specialized testing. The higher CBG concentrations in late-pregnant mice (which results in higher total corticosterone concentrations) also confounds interpretation of studies in which the magnitude of the basal or stimulated release of corticosterone in pregnant

mice is compared to the basal or stimulated release of corticosterone in cycling mice.

To explore an alternative to hormone measurements, we determined if acute activation of the HPA and SNS systems in late-pregnant mice could be assessed by measuring changes in core body temperature and if stress hyporesponsiveness of late pregnancy would manifest as a blunted rise in core body temperature.

OXT is known to attenuate the stress response and its release is increased in late pregnancy (Neumann et al., 1998). Others and we have questioned if OXT plays a role in stress hyporesponsiveness of late pregnancy. Douglas et al. (2003) reported that acutely administering an OXT antagonist intracerebroventricular (i.c.v.) did not prevent stress hyporesponsiveness in late-pregnant mice (as measured by ACTH or corticosterone release after exposure to a novel environment). Thus, short-term blockade of OXT pathways did not prevent the attenuated HPA response to stress in late-pregnant mice. The present set of experiments addressed this same question by comparing SIH in WT and OXTKO mice, in which functional OXT pathways are absent throughout the lifespan of the animal.

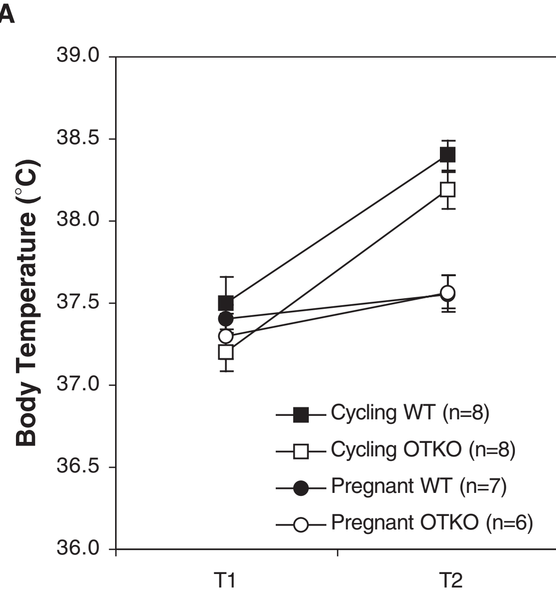
Nulliparous cycling mice (two to three per cage) were housed with a male mouse for 72 h to obtain pregnant mice. The day following this period (designated day 0 ± 3 of pregnancy) mice were transferred to single housing and abdominal girth was monitored. Mice were studied at estimated day 16–17 of pregnancy (parturition in our colony typically occurs on day 19–20 post-mating). After completion of the study, mice were sacrificed by CO_2 inhalation and the uterus dissected to verify pregnancy. All pregnant mice that were used in the analysis were verified to have pups.

We compared the rise in core body temperature following transfer to a metabolic cage in late-pregnant mice (6 OXTKO and 7 WT). Basal core temperature (T1) in pregnant mice of both genotypes was not different, and was not different from cycling female mice (Fig. 2). The rise in core temperature of pregnant mice was blunted compared to virgin (cycling) mice but to the same degree in both genotypes (Fig. 2, $p < 0.05$).

Ingestive behaviour and stress-induced hyperthermia in male mice pre-treated with a CB1R antagonist/inverse agonist

Cannabinoids exert complex effects upon the HPA axis (for a review, see Pagotto et al., 2006). Most studies suggest that endogenous cannabinoid-signalling pathways, similar to OXT-signalling pathways, attenuate stress-induced increases in corticosterone secretion. Cannabinoids were reported to suppress synaptic excitation of a variety of hypothalamic paraventricular (PVN) neurons, including CRH and OXT in vitro (Di et al., 2003). Consistent with the in vitro data of an inhibitory effect of endogenous cannabinoids on the HPA axis, basal corticosterone concentrations in male mice were greater 1 h after systemic injection of a selective CB1R 1 receptor antagonist/inverse agonist (SR141716, rimonabant), versus vehicle (Patel et al., 2004). Pre-injection of mice with CB1R antagonist 1 h prior to a 30 min restraint stress also heightened corticosterone release compared to vehicle-injected mice (Patel et al., 2004). Fos activation in the PVN accompanied the rise in corticosterone, but only in restrained and CB1R antagonist-treated mice, suggesting an inhibition of stress-induced activation of CRH neuron activity by endogenous cannabinoids (Patel et al., 2004). The data are consistent with studies supporting suppression of the synaptic excitation of CRH neurons by endogenous cannabinoids.

CB1R antagonists also decrease food intake (see review by Pagotto et al., 2006), presumably via interrupting central cannabinoid systems. Therefore, inhibition of feeding can be monitored to determine if a test dose of an administered CB1R antagonist is appropriate for blockade of central CB1 receptors. In the present studies, we first monitored the effects of the CB1R antagonist AM251 upon the intake of chow or a palatable lipid emulsion (Intralipid) before using the dose in stress studies. This afforded us the opportunity to record the effects of AM251 upon ingestive behaviours in mice that lack OXT pathways, given the hypothesized role that OXT-signalling pathways play in feeding behaviours (Arletti et al., 1989, 1990).



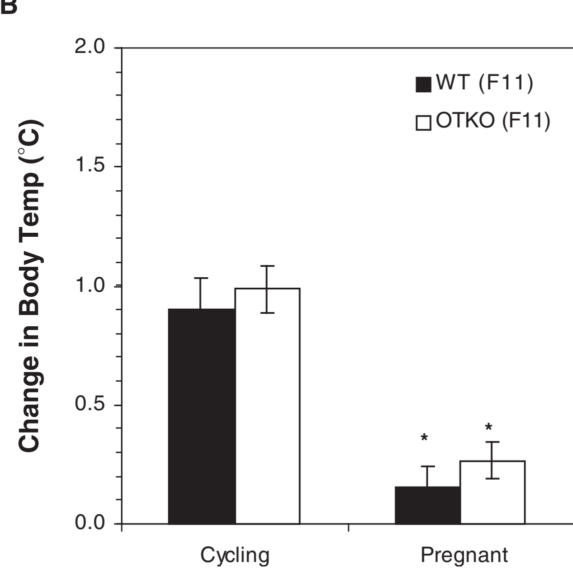


Fig. 2. (Panel A) Comparison of body temperature before, T1, and after, T2, stress in cycling and pregnant wild-type (WT) and oxytocin knockout (OTKO) mice. T1 temperatures were not different in cycling and pregnant mice. However, the T2 temperature of pregnant mice was significantly different than cycling mice ($p<0.05$). (Panel B) There were no genotypic differences in the stress-induced change in body temperature (T2−T1). However, the change in temperature induced by stress was significantly attenuated in the pregnant mice ($p<0.05$). The asterisks in Panel B indicate significant differences between cycling and pregnant mice.

AM251 is closely related in structure and activity to the CB1R antagonist SR141716, rimonabant, that was reported to enhance basal and post-stress plasma corticosterone concentrations when administered to mice in doses of 1 or 5 mg/kg (Patel et al., 2004). Rimonabant also decreases intake of food and palatable nutritive solution (Arnone et al., 1997). We therefore tested AM251

for effects upon feeding and stress in mice of both genotypes.

Effect of CB1R antagonist on ingestion

AM251 (3 mg/kg) was injected intraperitoneal (i.p.) in male WT and OXTKO mice (eight per genotype, F11 generation) using a study design whereby each mouse served as its own control. During the first phase of the study, four mice of each genotype were administered vehicle or AM251 at 17:00 h and hourly intake of food and a palatable liquid nutrient emulsion (Intralipid) was recorded until 23:00 h, then again at 09:00 h the following morning. Intake of food and Intralipid emulsion was recorded daily for 2 days before and 2 days after the injections. For this experiment, mice were maintained on powdered food, which allowed for accurate recording of food intake (Mantella et al., 2003b). A week later, the study was repeated. Mice that had received AM251 in the first phase received vehicle in the second phase of the study and mice that had received vehicle in the first phase of the study received AM251 in the second phase.

Figure 3A illustrates hourly intake of Intralipid in mice treated with vehicle or AM251. Intake of Intralipid was decreased in mice treated with AM251 compared to vehicle, but to the same degree in each genotype. Daily 24 h intake of food and Intralipid was reduced in mice administered AM251 compared to vehicle or baseline data, $p<0.05$, but to the same degree in both genotypes (Fig. 3B).

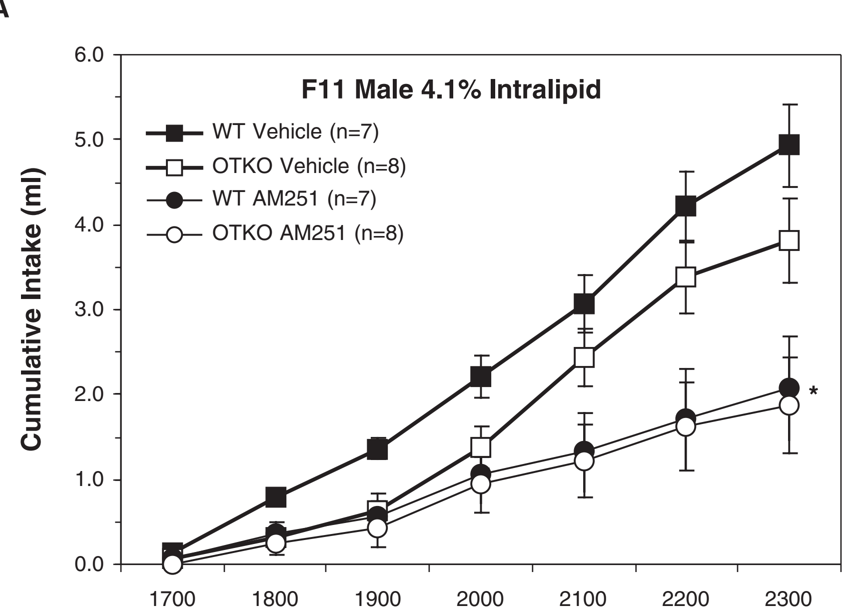
Effect of CB1R antagonist upon stress-induced hyperthermia in male mice exposed to a novel environment

Male OXTKO ($n = 8$) and WT ($n = 7$) mice from the F11 generation were single housed for 7 days prior to the start of SIH studies. Mice were injected with AM251, 3 mg/kg, i.p., and 1 h later rectal temperature was recorded before and 10 min after transfer to a metabolic cage. Two weeks later, the same mice were injected with vehicle and 1 h later core body temperature was recorded before and 10 min after transfer to a metabolic change. Basal core temperature (T1) differed among the mice but was not influenced by the treatment (Fig. 4). WT mice in this study had higher basal temperature than OXTKO mice during the AM251- and vehicle-treated phases of the study (Fig. 4). However, the peak temperature achieved did not differ between genotype or treatment group (Fig. 4). The rise in core body temperature was not different between vehicle and AM251 treatment in either genotype (Fig. 4). Because of the lower basal temperature in WT versus OXTKO mice, the change in temperature was greater ($p<0.05$) in WT than OXTKO mice.

Summary and implications of findings and future directions

OXTKO mice have been used for studying the proposed central effects of OXT. There is abundant literature implicating OXT in the responses to stress, anxiety and ingestive behaviour, including studies using this mouse model. Our results indicate that the combined effect of measuring rectal temperature, handling and transfer to a novel environment induces SIH in male and female WT and OXTKO mice. These data are consistent with previous research on SIH in mice, whether group or single housed. SIH was ineffective as a means to consistently identify the stress hyperresponsiveness of OXTKO mice and the reasons for this are discussed below. However, a novel finding from our study is that SIH can be used to identify the stress-hyporesponsive period of late pregnancy. Late-pregnant mice confronted with the same stress as cycling mice had comparable basal body temperatures but markedly blunted rises in core body temperature.

SIH is a useful test across species and among various mouse strains (Bouwknecht et al., 2007). SIH is also reported to be more robust across various mouse strains than exploratory-based behavioural tests, and can be used repeatedly without adaptation (Bouwknecht et al., 2007). We found that mice of both genotypes increased core body temperature following transfer to a metabolic cage, a manoeuvre that also triggers release



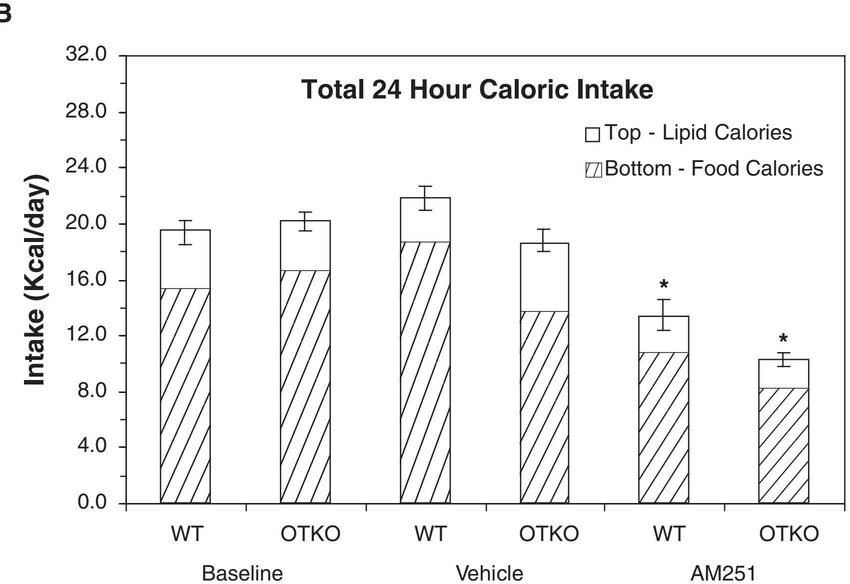


Fig. 3. (Panel A) Comparison of the cumulative hourly intakes of 4.1% Intralipid solution in male wild-type (WT) and oxytocin knockout (OTKO) mice treated with vehicle or the CB1 receptor antagonist, AM251, 3 mg/kg, i.p. The asterisk indicates that treatment with AM251 caused a significant decrease in the intake of Intralipid as evidenced by the downward shift of the cumulative intake curves ($p<0.05$). There were no differences in intakes between genotypes following vehicle or AM251 treatment. (Panel B) Twenty-four-hour caloric intake of mice treated with either vehicle or AM251. Bars are divided into two parts that indicate the calories that were ingested as solid food or lipid. AM251 caused significant decreases in total calorie intake of similar magnitude in both genotypes. The asterisks in Panel B indicate a significant difference between intake in mice treated with AM251 versus intake in mice treated with vehicle or baseline intake ($p< 0.05$).

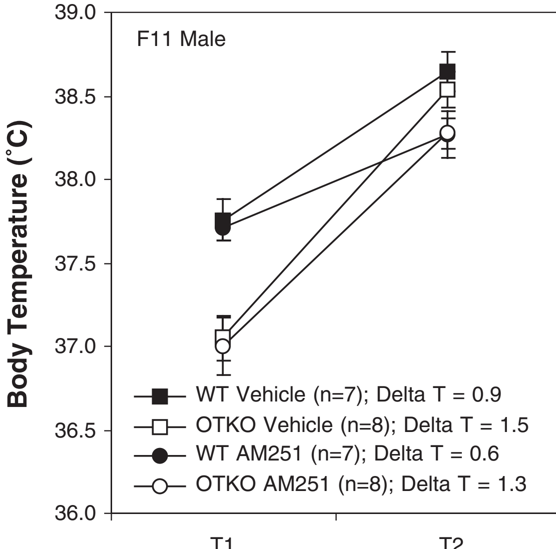


Fig. 4. Comparison of body temperature before, T1, and after, T2, stress in male wild-type (WT) and oxytocin knockout (OTKO) mice treated with vehicle or the CB1 receptor antagonist, AM251, 3 mg/kg, i.p. T1 temperatures were significantly lower in OTKO than WT mice ($p < 0.05$). There were no genotypic differences in the stress-induced change in body temperature (T2−T1). However, because of the lower baseline, T1, temperature in OTKO than WT mice, the change in temperature (Delta T) was significantly greater in OTKO than in WT mice ($p < 0.05$).

of corticosterone. Because female OXTKO mice hypersecrete corticosterone following certain stresses (Mantella et al., 2004, 2005) and demonstrate more anxiety-related behaviour in an EPM (Mantella et al., 2003a) than WT mice, we anticipated a heightened temperature response in OXTKO mice. Bouwknecht et al. (2007) indicate that SIH is an excellent test to identify anxiolytic actions of drugs. The anxiolytic effect is reflected as a blunting of the rise in core body temperature. On the other hand, SIH is limited in the opposite direction, namely identifying the anxiogenic effects of drugs or genetic manipulations (Bouwknecht et al., 2007). Drugs that are known anxiogenics or high-anxiety strains of mutant mice could not be readily identified by SIH, despite the fact that such conditions would predict a higher than anticipated temperature response. Our present observations also support these findings. We did not consistently identify greater temperature increases in OXTKO male or female mice compared to WT mice, although in a pilot study we had previously reported heightened temperature increases in a small cohort of female OXTKO mice ($n = 5$) compared to WT mice ($n = 5$) (Amico et al., 2004).

SIH was not enhanced in our mice following administration of a CB1 antagonist, which has been previously reported to induce increases in basal and stress-induced corticosterone release in mice (Patel et al., 2004). One possible explanation for our observations is that the intensity of the stress applied to invoke SIH in the present study was insufficient compared to the stress used to evoke corticosterone release in the previous studies (restraint). van der Heyden et al. (1997) reported that the amplitude of the hyperthermic response is dependent on the intensity of the stress. The procedure itself, which consists of handling and insertion of a rectal probe, will evoke a rise in temperature and release of corticosterone. However, when the procedure is combined with additional components, such as transfer to a novel cage, the amplitude of SIH is increased. This indicates that the SIH procedure is not restricted by a ceiling effect on the stressed temperature. Future studies can be directed at recording temperature change following more intense or different types of stressors. Additionally, recording only a single temperature 10 min after the stress (the standard way the test is conducted) may limit the ability to detect significant changes. Perhaps a better way to monitor temperature changes will be to equip mice with 24 h temperature probes so that an integrated (area under the curve) response can be measured. Thus, the failure to consistently identify differences in the SIH response of OXTKO versus WT mice or vehicle-treated versus CB1R antagonist-treated mice may be due to an inherent limitation of the technique currently used to measure SIH.

Lastly, CB1R antagonists are known to decrease food intake in a variety of mammals (Pagotto et al., 2006). In the present study, blockade of CB1R pathways decreased intake of both food and a palatable liquid emulsion in mice of both genotypes. Arletti et al. (1989, 1990) reported that pharmacological doses of peripherally

administered synthetic OXT inhibited food ingestion in rats. In addition, cannabinoid and OXT pathways are known to interact (Di et al., 2003; Oliet et al., 2007). Verty et al. (2004) suggested that OXT and cannabinoid-signalling pathways interact to decrease ingestive behaviour in rats. As such we conducted experiments to examine a possible interplay of the two systems upon ingestive behaviour in mice. If cannabinoid-signalling pathways regulate ingestion independent of OXT-signalling pathways, then the decrement in ingestion should be comparable in both OXTKO and WT mice. On the other hand, if OXT-signalling pathways are necessary for the anorexigenic effect of CB1 antagonist, then the CB1 antagonist will only be effective in WT, but not OXTKO, mice. If OXT facilitates CB1R antagonism, then intake will decrease in both genotypes but to a lesser degree in OXTKO than WT mice. Because we identified no genotypic differences in the ingestion of either standard mouse chow or a palatable liquid fat emulsion, we conclude that the anorexigenic actions of the CB1 antagonist can occur independent of OXT-signalling pathways in mice. OXT-signalling pathways are also not essential in mice for the regulation of basal food intake or caloric balance (Mantella et al., 2003b) nor the feeding that occurs after an overnight fast (Mantella et al., 2003b; Rinaman et al., 2005). OXT-signalling pathways are also not essential for the anorexigenic effects of exogenously administered cholecystokinin or d-fenfluramine (Mantella et al., 2003b). On the other hand, OXT-signalling pathways appear to be important for the anorexia that accompanies dehydration (Rinaman et al., 2005). Future studies are needed to elucidate the manner by which OXT pathways may be exerting these differential effects upon feeding behaviour.

Abbreviations

ACTH	adrenocorticotropin hormone
CB1R	cannabinoid 1 receptor
CBG	corticosterone-binding globulin
CRH	corticotropin-releasing hormone
EPM	elevated plus maze
HPA	hypothalamic-pituitary-adrenal
HZ	heterozygote
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
OXT	oxytocin
OXTKO	oxytocin knockout
PVN	paraventricular nucleus of the hypothalamus
SIH	stress-induced hyperthermia
SNS	sympathetic nervous system
T1 or T2	temperature 1 or 2
WT	wild-type

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 6

The role of the vasopressin 1b receptor in aggression and other social behaviours

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Abstract: While the importance of vasopressin (Avp) in the neuroendocrine regulation of behaviour is clear, most of Avp's effects on behaviour have been linked to its action via its 1a receptor (Avpr1a) subtype. There is, however, emerging evidence and cross-species consensus that the vasopressin 1b receptor (Avpr1b) is also important in mediating the effects of Avp on behaviour. The Avpr1b is highly expressed in the anterior pituitary where it is thought to play a role in the neuroendocrine response to stress. The Avpr1b is also prominently expressed in the pyramidal cells of the CA2 hippocampal area. Interestingly, in mice, Avpr1b mRNA within the pyramidal neurons of the CA2 field is unaffected by restraint stress or adrenalectomy. Avpr1b knockout mice (−/−) have provided strong, consistent evidence that the Avpr1b plays a critical role in the regulation of social behaviour. Avpr1b$^{-/-}$ mice display reduced levels of social forms of aggression, reduced social motivation and impaired social memory (including the Bruce effect). Avpr1b$^{-/-}$ mice, however, have normal main olfactory ability, spatial memory and defensive and predatory behaviours. Mice lacking a functional accessory olfactory system display many of these same behavioural deficits, suggesting that Avpr1b$^{-/-}$ mice may have a deficit in the processing, perception and/or integration of olfactory stimuli detected by the accessory olfactory system. We suggest that the role of the Avpr1b is to couple socially relevant accessory olfactory cues with the appropriate behavioural response. Furthermore, given its prominence in the CA2 field of the hippocampus, we hypothesize that Avpr1b may be important for the formation or recall of memories that have an olfactory-based social component.

Keywords: vasopressin; vasopressin 1b receptor; aggression; social memory; social recognition; social motivation; hippocampus

Introduction

Originally described in pituitary (Antoni, 1984; Jard et al., 1986; Arsenijevic et al., 1994) and subsequently cloned (Lolait et al., 1995), the vasopressin (Avp) 1b receptor (Avpr1b) is found in several brain areas and peripheral tissues

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DOI: 10.1016/S0079-6123(08)00406-8

(Arsenijevic et al., 1994; Lolait et al., 1995; Young et al., 2006). Within the anterior pituitary the Avpr1b facilitates the release of adrenocorticotropic hormone (ACTH) from the corticotropes (Jard et al., 1987; Antoni, 1993), thus helping mediate the effects of Avp on the hypothalamic–pituitary–adrenal axis, i.e., the mammalian stress axis (Volpi et al., 2004). In the periphery, *Avpr1b* mRNA is found in tissues including kidney, thymus, heart, lung, spleen, uterus and breast (Lolait et al., 1995), although its role in these tissues remains unclear. It is only relatively recently that *Avpr1b* transcripts as well as Avpr1b immunoreactive cell bodies have been found in the rat brain, including in the olfactory bulb, piriform cortical layer II, septum, cerebral cortex, hippocampus, paraventricular nucleus, suprachiasmatic nucleus, cerebellum and red nucleus (Lolait et al., 1995; Saito et al., 1995; Vaccari et al., 1998; Hernando et al., 2001; Stemmelin et al., 2005). It should be noted, however, that Avpr1b distribution has not been mapped by receptor autoradiography due to the lack of a specific radiolabeled ligand. A recent study using in situ hybridization histochemistry with more specific probes on mouse, rat and human tissues found prominent *Avpr1b* expression in the hippocampal CA2 field pyramidal neurons (Young et al., 2006).

The Avpr1b knockout mouse (Avpr1b$^{-/-}$) was developed prior to any Avpr1b-specific pharmacology and has provided critical insights into the roles of the Avpr1b in the mouse, and possibly other species. While pharmacologically 'clean' compounds have continued to be somewhat elusive, the available pharmacological data are consistent with our observations in Avpr1b$^{-/-}$ mice (Blanchard et al., 2005; Griebel et al., 2005). Originally described by Wersinger et al. (2002, 2004), Avpr1b$^{-/-}$ mice, compared to wildtype controls, have reduced aggression and investigation of social cues as well as mild deficits in social memory. Taken together, these findings suggest that absence of the *Avpr1b* gene results in impairments that affect social behaviours more than other behaviours. The Avpr1b is also important for normal responses to some acute and chronic stressors (for review see Serradeil-Le Gal et al., 2005). While genetic disruption of the *Avpr1b* fails to affect measures of anxiety-like behaviour, depression-like behaviour (Wersinger et al., 2002; Caldwell et al., 2006) or the corticosterone response to acute restraint stress (Lolait et al., 2007a); its disruption attenuates the corticosterone response to forced swim, acute insulin treatment, acute immune stress and ethanol intoxication (Tanoue et al., 2004; Lolait et al., 2007a, b). Avpr1b$^{-/-}$ mice also demonstrate a blunted ACTH response under chronic stress conditions (Lolait et al., 2007a) and pharmacological blockage of Avpr1b receptors reduces ACTH secretion (Serradeil-Le Gal et al., 2002). While the role of the Avpr1b in the mediation of the stress axis and other physiological responses (Oshikawa et al., 2004; Itoh et al., 2006; Fujiwara et al., 2007) are interesting and active areas of research, this chapter will focus on the contributions of the Avpr1b to the regulation of social behaviour.

Avpr1b and aggression

The *Avpr1b* gene is essential for the normal expression of aggressive behaviour. Furthermore, the Avpr1b is critical for the initiation of offensive behaviour, but only toward a conspecific. Avpr1b$^{-/-}$ mice have longer attack latencies and fewer attacks toward an intruder compared to wildtype controls in a resident–intruder test (Wersinger et al., 2002, 2006) (Fig. 1). Disruption of the Avpr1b does not, however, result in a global deficit in all aggressive behaviour. Several experiments support this conclusion. When attacked by a stimulus animal, Avpr1b$^{-/-}$ mice exhibit normal defensive avoidance behaviours (Wersinger et al., 2006). Further, experience modulates their aggression, as repeated testing results in increased aggression, though, never to the levels observed in wildtype controls (Wersinger et al., 2006).

One of the most intriguing aspects of the aggression phenotype found in Avpr1b$^{-/-}$ mice is that it is specific to social forms of aggression. When given the opportunity to predate a cricket, Avpr1b$^{-/-}$ mice display latencies to attack comparable to wildtype littermates (Fig. 2). In a competitive aggression test, where the animals are food deprived, Avpr1b$^{-/-}$ mice show higher levels of

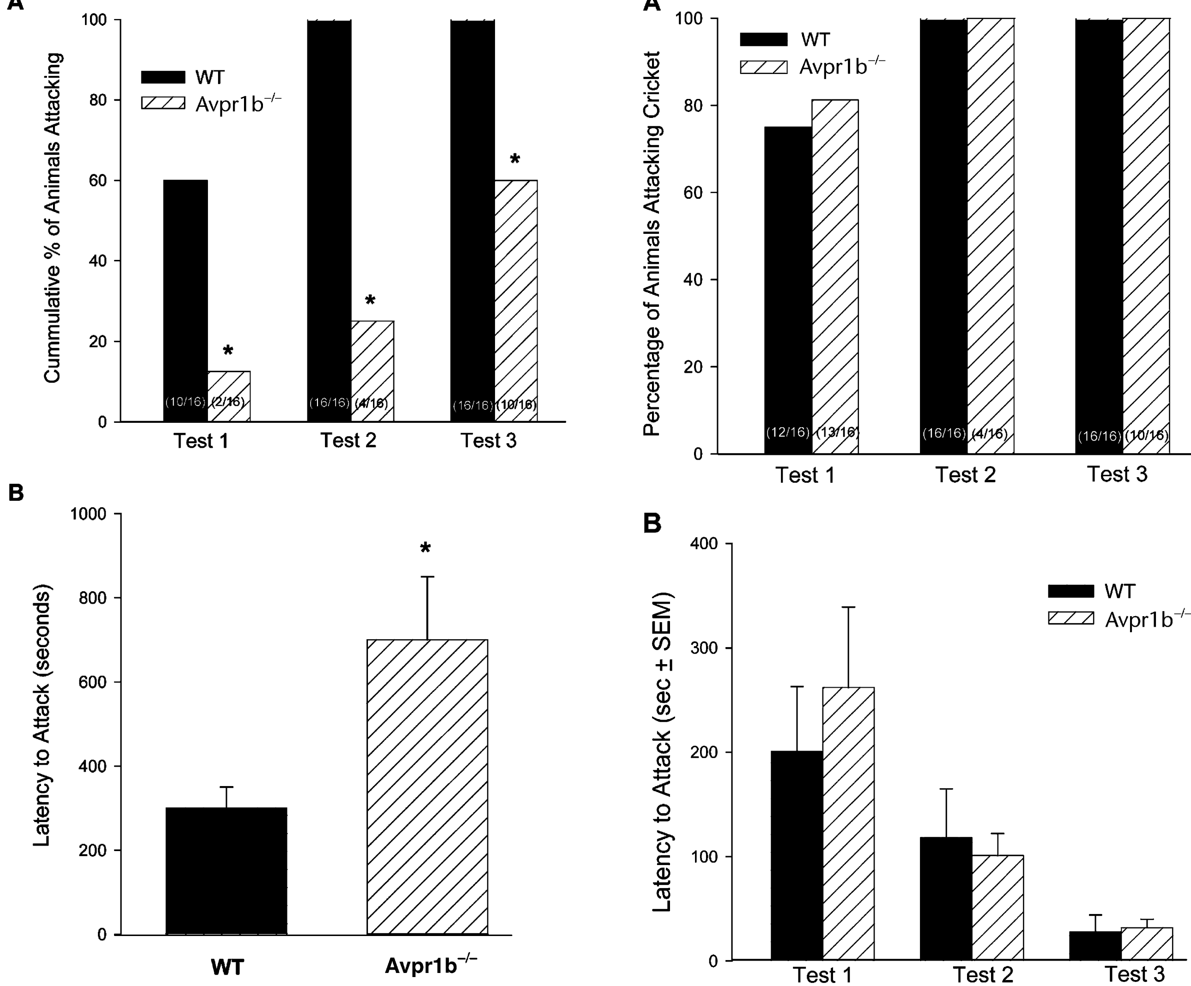

Fig. 1. Reduced aggression in Avpr1b$^{-/-}$ male mice compared to WT mice in a resident–intruder test. (A) A significantly lower percentage of Avpr1b$^{-/-}$ males display aggressive behaviour than WT males across three test sessions. (B) Avpr1b$^{-/-}$ males have longer latencies to attack compared to WT males ($^* = p<0.05$).

Fig. 2. Predatory aggression is preserved in Avpr1b$^{-/-}$ male mice. (A) WT and Avpr1b$^{-/-}$ male mice do not differ in the percentage of animals that will attack a cricket. (B) The latencies to attack the crickets do not differ between the two genotypes. The latencies decrease with experience with crickets.

aggression than during non-fasting conditions, but still not to the level found in wildtype animals (Wersinger et al., 2006). The preservation of predatory behaviour unequivocally shows that Avpr1b$^{-/-}$ mice are capable of perceiving, identifying and attacking a stimulus. From previous work we have also established that Avpr1b$^{-/-}$ mice are able to, at least on some level, respond to a novel conspecific, as confrontation with one results in increases in Fos immunoreactivity in olfactory areas and elevated cortisol (Wersinger et al., 2002).

Avpr1b and social memory

The ability of an organism to recognize individuals or social situations is critical in the determination of what behaviours will be displayed. In mice, one

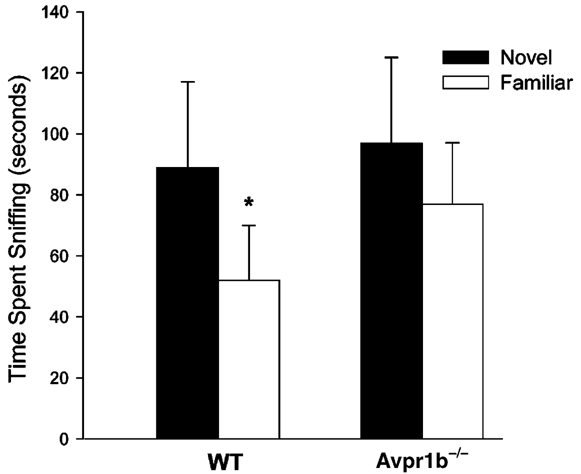

Fig. 3. Social recognition is impaired in Avpr1b$^{-/-}$ male mice compared to WT mice. With a 30-min interval between exposures, Avpr1b$^{-/-}$ males do not appear to recognize a familiar animal. (* = significantly less than the first exposure, $p<0.05$.)

way to test social memory is to use a social recognition test in which, based on sniff time, a mouse demonstrates the ability to discriminate familiar from novel individuals. Avp is important for individual recognition, in particular through the androgen-dependent vasopressinergic projections from the medial amygdala and bed nucleus of the stria terminalis to the lateral septum (De Vries et al., 1984; Mayes et al., 1988; Bluthe et al., 1990, 1993). Although previous work has focused primarily on the vasopressin 1a receptor (Avpr1a) (Engelmann and Landgraf, 1994; Landgraf et al., 1995, 2003; Bielsky et al., 2003) [however, see Bielsky et al., 2005; Wersinger et al., 2007], work in our laboratory has identified impairments in social recognition in male Avpr1b$^{-/-}$ mice (Fig. 3). These findings demonstrate that vasopressinergic regulation of social recognition is mediated not only by the Avpr1a, but also the Avpr1b.

Another form of social memory is pregnancy block (the Bruce Effect) in female mice. The Bruce Effect, is the pheromonally mediated pregnancy termination of a recently mated female by exposing her to chemosensory cues from an unfamiliar male (Bruce, 1959; Bruce and Parrott, 1960). The Bruce effect is mediated by the accessory olfactory system since removal of the vomeronasal organ, but not the main olfactory epithelium, eliminates pregnancy block (Bellringer et al., 1980). When Avpr1b$^{-/-}$ females are tested for the Bruce Effect they fail to terminate their pregnancies in the presence of an unfamiliar male (Wersinger et al., 2008). This suggests that Avpr1b$^{-/-}$ females have a deficit in their olfactory, or more specifically, their accessory olfactory, memory since they are unable to discriminate familiar from unfamiliar. Our hypotheses about the role of the Avpr1b in social memory are presented in the discussion.

Avpr1b and social motivation

To characterize more fully the social deficits found in Avpr1b$^{-/-}$ mice, we tested their motivation to interact with socially salient stimuli. Described in Wersinger et al. (2004), Avpr1b$^{-/-}$ mice and wildtype mice were tested for bedding preferences. The mice were given three different preference tests: (1) female-soiled bedding versus male-soiled bedding; (2) female-soiled bedding versus clean bedding; (3) male-soiled bedding versus clean bedding. During each of these tests, the percent of total investigation time spent at each stimulus was determined. The expectation was that males that were 'socially motivated' would spend more time investigating female-soiled bedding over male-soiled bedding, and either would be preferable over clean bedding. This was the case for the male wildtype mice; however, as shown in Fig. 4, male Avpr1b$^{-/-}$ mice spend a similar about of time investigating both male- and female-soiled bedding, male-soiled bedding and clean bedding and female-soiled bedding and clean bedding. In addition, Avpr1b$^{-/-}$ mice spend less time overall investigating the bedding compared to wildtype control. These differences could be easily explained if Avpr1b$^{-/-}$ mice have impairments in their olfactory ability. However, this is unlikely to be the case. In a hidden cookie test, Avpr1b$^{-/-}$ mice locate a cookie hidden under bedding as quickly as their wildtype littermates (Wersinger et al., 2002); the hidden cookie test is an excellent screen to detect major deficits in olfactory function. As olfactory function is absolutely critical to

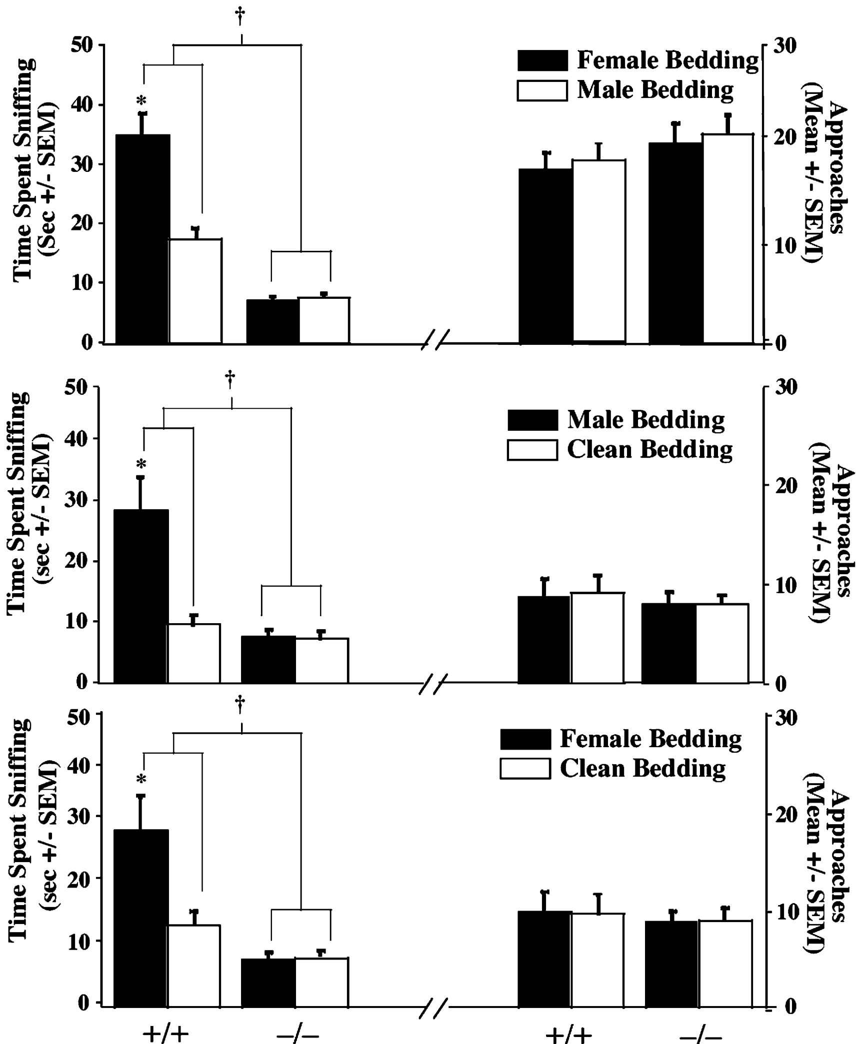

Fig. 4. Chemoinvestigatory behaviour of soiled bedding by male WT (+/+) and Avpr1b$^{-/-}$ (–/–) mice. Amount of time investigating (seconds ± SEM) (left side of panels) and number of approaches (± SEM) (right side of panels) to female-soiled bedding versus male soiled-bedding (top panel), male soiled bedding versus clean bedding (middle panel) or female soiled bedding versus clean bedding (bottom panel) are shown. In all tests male WT mice spent significantly greater amount of time investigating bedding compared to Avpr1b$^{-/-}$ mice ($^{\dagger} = p < 0.01$). Only male WT mice showed preferences for one stimulus over the other ($^{*} = p < 0.01$).

normal social behaviour in the mouse, we felt it was necessary to assess olfactory ability as thoroughly as possible. Therefore, we supplemented the hidden cookie test using an operant conditioning task patterned after that used by Slotnick and Nigrosh (1974). This operant conditioning task uses thirst as a motivation thereby eliminating changes in social motivation as a potential confound. We determined that Avpr1b$^{-/-}$ mice are able to detect dilute urine and to discriminate male urine from female urine (Wersinger et al., 2002, 2004). Taken together, our findings suggest that the deficit Avpr1b$^{-/-}$ mice display in the previously mentioned bedding preference tests is one of social motivation and not related to olfactory deficits. Future studies will focus on testing the hypothesis that social motivation is reduced in Avpr1b$^{-/-}$ mice.

Conclusions

Over the past several years, we have laid the foundation necessary to study the role of the Avpr1b in the regulation of social behaviour. We have demonstrated that the deficits in behaviour all share one common feature — modulation by accessory olfactory information. Mapping the Avpr1b in the mouse was challenging and until recently (Young et al., 2006) we were not able to localize the receptor's expression. While there is much work to be done to understand the role of the Avpr1b in the regulation of social behaviour, future work will focus on the contribution of specific brain regions expressing the Avpr1b in social behaviour. The receptor's prominence in the CA2 field of the hippocampus was unexpected but is very tantalizing. The CA2 field has some unique features, including a resistance to damage following head trauma (Maxwell et al., 2003) and intractable epilepsy (Fried et al., 1992; Mathern et al., 1995; El Bahh et al., 1999). It is also the only region of the hippocampus that receives input from the hypothalamic supramammillary nucleus (Vertes and McKenna, 2000). The CA2 field also receives direct input from the entorhinal cortex, amygdala and, perhaps, the dentate gyrus (Bartesaghi et al., 2006). The amygdala receives olfactory information from both the main and accessory olfactory bulbs. In addition, it appears that CA2 can provide feedback inhibition on itself and to CA3 and feedforward inhibition to CA1 (Mercer et al., 2007). These characteristics, as well as others, suggest that the CA2 field is not simply an extension of the CA3 field of the hippocampus. In addition, lesions of the hippocampus that include the CA2 field result in behavioural changes similar to those observed in Avpr1b$^{-/-}$ mice, i.e. reduced aggression and impaired social recognition (Ely et al., 1977; Maaswinkel et al., 1996). It therefore seems plausible that the presence of the Avpr1b in this region may explain the behavioural deficits found in Avpr1b$^{-/-}$ mice.

One explanation for the reduced aggression and impaired social recognition found in Avpr1b$^{-/-}$ mice, with seemingly normal sensory perception, may be an inability to associate the context with the social experience. This disconnect would result in an incorrect behavioural output. Given the prominence of the Avpr1b in the CA2 field of the hippocampus, we hypothesize that Avpr1b may be important to the formation of memories that are accessory olfactory system-based. If this is the case, then the Avpr1b may be helping to encode the social context and perhaps even stimulate the retrieval of a previous social memory. It is tempting to speculate that the hippocampus has 'social' cells that respond to similar social settings in an analogous fashion to place cells (Smith and Mizumori, 2006). The Avpr1b would then be critical for their proper functioning.

Developmental compensation is a limitation of all traditional knock-out models. In fact, we believe that part of the reason we failed to detect major deficits in vasopressin 1a knockout mice is that the nervous system is able to successfully compensate for the loss of the gene. It is certainly possible that our behavioural findings in Avpr1b$^{-/-}$ mice are related more to developmental compensation (e.g., in the serotonin system) and less to the specific activity of the Avpr1b. Pharmacological evidence, however, supports a role for the Avpr1b in the adult animals. In hamsters, oral administration of an Avpr1b antagonist reduces aggression (Blanchard et al., 2005), suggesting not only that developmental compensation is unlikely to be the mechanism by which the Avpr1b affects aggression, but also that the role of the Avpr1b is conserved across distantly related rodent species. The presence of the Avpr1b within the CA2 field of hippocampus across mouse, rat and human suggests that whatever its role may be, it appears to be evolutionarily conserved. Future work will focus on the contribution of the CA2 field of hippocampus to the behavioural phenotype observed in Avpr1b$^{-/-}$ mice.

Abbreviations

ACTH	adrenocorticotropic hormone
Avp	vasopressin
Avpr1a	vasopressin 1a receptor
Avpr1b	vasopressin 1b receptor
Avpr1b$^{-/-}$	vasopressin 1b receptor knockout

Acknowledgement

This work was supported by the NIMH Intramural Research Program (Z01-MH-002498-17).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 7

Behavioural studies using temporal and spatial inactivation of the oxytocin receptor

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Abstract: Oxytocin (Oxt), synthesized in magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei for transport to and release from the posterior pituitary, is released during parturition and is essential for lactation. Lesser amounts of Oxt are made by smaller cells of the PVN and a few other forebrain nuclei and released into the central nervous system (CNS) to influence various other behaviours. In both the periphery and CNS, Oxt actions are transduced by the oxytocin receptor (Oxtr). Previously, it has been reported that $Oxt^{-/-}$ (knockout, KO) mice show a failure of milk ejection and thus are incapable of rearing their offspring. Unexpectedly, these mice have largely normal reproductive and maternal behaviours, perhaps due to compensatory mechanisms through activation of the Oxtr by vasopressin or through development. To examine the specific roles of the Oxtr during development and in particular brain areas, we created conditional $Oxtr^{-/-}$ mice in which we could control the spatial and temporal inactivation of the *Oxtr*. We flanked the neomycin-resistance selectable marker in an *Oxtr* intron with FRT sites to enable its removal using FLP recombinase. Coding sequence within exons 2 and 3 was flanked by two loxP sites enabling subsequent inactivation of the gene by targeted expression of Cre recombinase. The first *Oxtr* KO lines we created have either total or relatively specific forebrain elimination. The latter was achieved by crossing the conditional *Oxtr* line with a transgenic line in which the *Camk2a* promoter drives expression of Cre recombinase to significant levels beginning 21–28 days after birth, thus eliminating potential compensation for a deleted *Oxtr* gene during early development. This Cre-expressing line also significantly spares the main olfactory bulb reducing the potential confound of an olfactory deficit. We have investigated various behaviours, most notably social recognition, in both *Oxtr* KO strains ($Oxtr^{-/-}$ and $Oxtr^{FB/FB}$).

Keywords: oxytocin; Oxtr; conditional; knockout; behaviour; transgenic; Camk2a; Cre

Introduction

The first gene knockout (KO) mouse created by homologous recombination was introduced in the late 1980s (Mansour et al., 1988; Thompson et al., 1989). This powerful technology has facilitated the study of various mammalian biological processes, from signal transduction to animal behaviour. The neurotransmitter oxytocin (Oxt) and its sole receptor, oxytocin receptor (Oxtr), have also been studied using the KO mouse model. Oxt is the most abundant hormone in the hypothalamus and

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DOI: 10.1016/S0079-6123(08)00407-X

is best known for its role in reproductive functions such as parturition and lactation (Gimpl and Fahrenholz, 2001). The majority of Oxt is synthesized in magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, and released from the posterior pituitary into the general blood circulation (Brownstein et al., 1980). Lesser amounts of Oxt are made by smaller cells of the PVN and a few other forebrain nuclei and released into the central nervous system (CNS) to influence behaviour. In both the periphery and CNS, Oxt actions are transduced by the Oxtr, which belongs to the G-protein-coupled receptor family and coupled with phospholipase C by interacting via $G_{\alpha q11}$ (Young and Gainer, 2003). In the mouse brain, the *Oxtr* gene is expressed in various regions including the hypothalamus, olfactory system, amygdala and hippocampus (Insel et al., 1991; Gould and Zingg, 2003), implicating the *Oxtr* in various functions throughout the brain. In this chapter, the behavioural functions of Oxtr through use of the conditional KO technology are discussed.

Oxt and *Oxtr* knockout mice

A large number of behavioural studies have utilized KO mouse models, with many focusing on Oxt and Oxtr. The first reports on $Oxt^{-/-}$ mice were made in 1996 by two independent groups, with the most notable deficits being an inability to lactate and support their own offspring (Nishimori et al., 1996; Young et al., 1996). In spite of the clear reproductive function of Oxt in milk production, $Oxt^{-/-}$ mice display essentially normal parturition and maternal behaviour with some exceptions, such as pup retrieval (Nishimori et al., 1996; Young et al., 1996). To explain these results, it was hypothesized that a functional redundancy through the activation of the *Oxtr* by vasopressin or through developmental compensation occurs (Caldwell and Young, 2006). However, $Oxtr^{-/-}$ mice generated by Takayanagi et al. (2005) show no obvious deficits in mating and parturition. Pups show increased exploration and decreased ultrasonic vocalizations consistent with less stress- or anxiety-like behaviour. These mice also display increased aggression attributed to lack of Oxtr activation by maternal Oxt prenatally (Takayanagi et al., 2005).

Additional studies in $Oxt^{-/-}$ and $Oxtr^{-/-}$ mice have found deficits in social memory (Ferguson et al., 2000; Takayanagi et al., 2005), increased anxiety (Mantella et al., 2003) and increased intake of sucrose (Amico et al., 2005).

Behavioural analysis using conditional knockout mice

Although the KO mice are great tools to study the function of the genes, the conventional KO technology has the following three limitations: (1) genes that are essential for development could lead to a lethal embryonic phenotype in the KO; (2) KO of a gene could show no phenotype due to functional redundancy; (3) KO of a widely expressed gene would not address its specific functions in different tissues (Gaveriaux-Ruff and Kieffer, 2007). In order to overcome these issues, Cre-loxP (Sternberg and Hamilton, 1981) and FLP-FRT (Broach and Hicks, 1980) systems have been applied to KO techniques. These site-specific recombinases (Cre and FLP) allow generation of temporally and/or spatially regulated KO mice (Fig. 1). Gene inactivation can be achieved through expression of Cre recombinase under the regulation of cell-type-specific promoters (Lewandoski, 2001). Early expression of Cre can cause functional inactivation in the same manner as conventional KO mice. Therefore, for behavioural studies, the use of late onset promoters is more useful to obtain the benefit of temporal regulation (Morozov et al., 2003; Gaveriaux-Ruff and Kieffer, 2007). In addition, brain and peripheral functions can interact and influence one another. For example, in the Oxt system, the inability of $Oxt^{-/-}$ mice to lactate affects their ability to display maternal behaviours.

Phenotypical implication of Oxtr conditional knockout mice

We generated both forebrain-specific *Oxtr* conditional KO ($Oxtr^{FB/FB}$) and total *Oxtr* KO ($Oxtr^{-/-}$) mouse lines. $Oxtr^{FB/FB}$ mice were made by crossing floxed Oxtr mice with transgenic mice in which

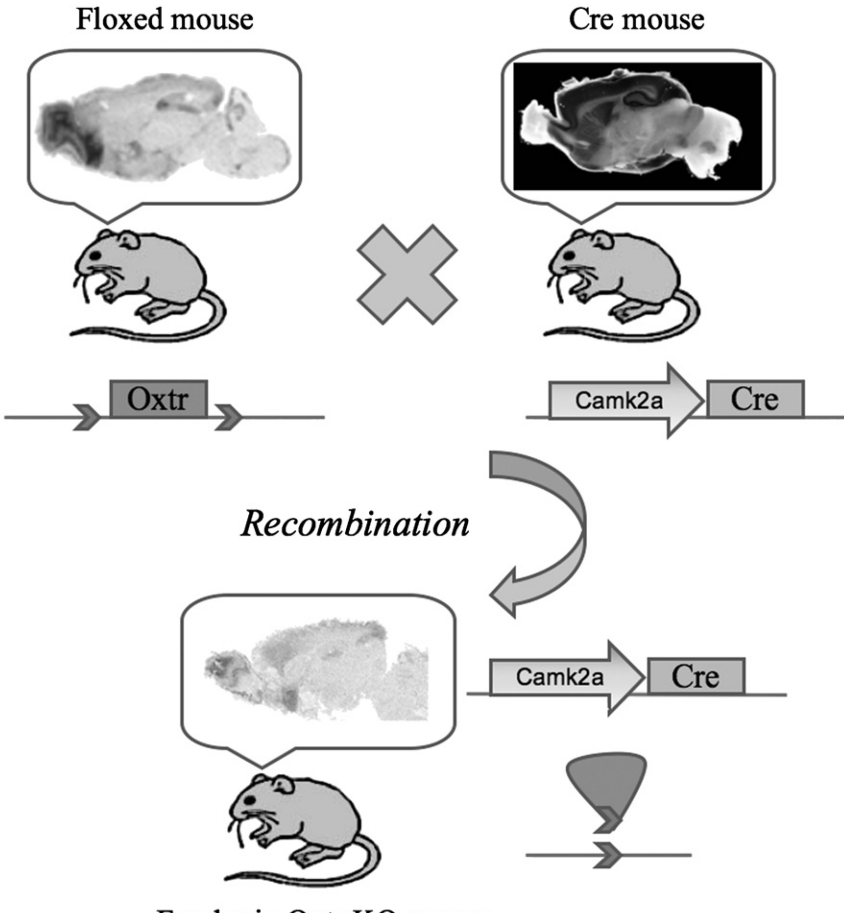

Fig. 1. Controlling the *Oxtr* gene expression by Cre-mediated DNA recombination. Coding sequence of the *Oxtr* gene allele that is flanked by loxP sites (floxed) is produced by homologous recombination. The *Camk2a*-Cre promoter drives Cre expression in transgenic mice relatively specifically in the forebrain. By crossing the floxed mice with the Cre transgenic mice, progeny are produced in which the conditional allele is inactivated only in the forebrain. Therefore, the *Oxtr* expression still remains in other tissues, such as the mammary glands. Cre recombinase recognizes two loxP sites (chevrons) and eliminates the floxed *Oxtr* exons.

Ca^{2+}/calmodulin-dependent protein kinase II alpha (*Camk2a*) promoter drives expression of Cre. This promoter allows the *Cre* gene to be expressed relatively specifically in the forebrain (Dragatsis and Zeitlin, 2000). Indeed, effective Cre levels appear between 21 and 28 days after birth, thus eliminating compensation for a deleted *Oxtr* gene during early development. This Cre-expressing line also significantly spares the main olfactory bulb reducing the potential confound of a deficit in olfaction, which is critical for rodent behaviours (Lee et al., 2008).

Both $Oxtr^{FB/FB}$ and $Oxtr^{-/-}$ mice show similar performances in assessments of general health, including reflexes, sensory and motor tests. The $Oxtr^{FB/FB}$ mice are able to lactate and display no deficits in maternal behaviour when left undisturbed. Since previously reported $Oxtr^{-/-}$ showed impaired maternal behaviours (Takayanagi et al., 2005), the different phenotypes between $Oxtr^{FB/FB}$ and $Oxtr^{-/-}$ mice may be due to the incomplete elimination of Oxtr in the brain regions that underlie expression of maternal behaviour or differences in testing conditions. Interestingly,

the *Oxtr*$^{FB/FB}$ and *Oxtr*$^{-/-}$ show differences in social recognition that we are currently investigating. Besides the phenotypes mentioned above, *Oxtr*$^{FB/FB}$ and *Oxtr*$^{-/-}$ mice show the same aspects of behaviour including general health and olfaction. Further in depth studies, including assessment of aggression, object memory and maternal behaviour in virgin females are progressing.

Future directions

Impairment of social recognition is strongly related to some neurodevelopmental disorders such as autism (Young et al., 2002). As mounting evidence exists suggesting an association between the Oxt system with autism in humans (Auranen et al., 2002; Shao et al., 2002; Wu et al., 2005; Jacob et al., 2007), the *Oxtr* KO mouse lines could provide useful model systems to study autism. Precise temporal and spatial regulation of Oxtr will allow finer investigations into the roles of Oxt and Oxtr in various behaviours. This precise temporal and/or spatial manipulation will be possible through the use of transgenic mice or lentiviruses expressing tetracycline-inducible Cre recombinase.

Abbreviations

Camk2a	Ca^{2+}/calmodulin-dependent protein kinase II alpha
CNS	central nervous system
KO	knockout
Oxt	oxytocin
Oxtr	oxytocin receptor
PVN	paraventricular nucleus
SON	supraoptic nucleus

Acknowledgement

This work was supported by the NIMH Intramural Research Programme (Z01-MH-002498-17).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 8

New aspects of oxytocin receptor function revealed by knockout mice: sociosexual behaviour and control of energy balance

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Abstract: To further define the function of the oxytocin receptor (OXTR) in vivo, we generated mice deficient in the Oxtr gene (*Oxtr−/−*). *Oxtr−/−* mice had no obvious deficits in fertility or sexual behaviour, but displayed several aberrations in social behaviours, including male aggression, and mother–offspring interaction. In addition, they showed novel physiological defects including obesity, and dysfunction in body temperature control when exposed to cold. We review here our new findings with *Oxtr−/−* mice, and introduce newly generated Oxtr-Venus knockin mice as a potential tool for examining molecular physiology of Oxtr-neurons.

Keywords: maternal behaviours; social discrimination; aggressive behaviours; thermo regulation; localization of oxytocin receptor expressing neurons

Oxytocin and oxytocin receptor

Oxytocin synthesis

Although occupying less than 1% of the whole brain volume in the case of the human, the hypothalamus is an important control centre that regulates metabolism, body temperature, heart beat rate, blood pressure, water and food intake and behaviour. These controls are generated in response to alterations in the outer and internal environment of the body and performed in part via the endocrine system and through the autonomic nervous system in response to physical and chemical signals. The neuropeptide oxytocin (OXT), with only nine amino acids (a.a.), was first identified as one of the prototypical hypothalamic hormones that is released into the bloodstream from the posterior pituitary. It is now known that OXT released within the brain acts as a neuromodulator/neurotransmitter through its actions on its G-protein coupled receptor (GPCR) (Landgraf and Neumann, 2004). OXT, which is synthesized as a 125 a.a. precursor

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DOI: 10.1016/S0079-6123(08)00408-1

and includes a neurophsyin, is synthesized primarily in magnocellular neurons located at the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. After processing by a protease and formation of an intramolecular disulfide bond, OXT is transported to the posterior lobe of the pituitary and secreted into the blood in response to various physiological stimuli, including vaginocervical and nipple stimulation. In addition to this hypothalamic source, OXT is also synthesized in various peripheral tissues and organs, including the uterine epithelium, ovary, testis, vascular endothelium cell and heart.

Oxytocin receptor

The receptor for oxytocin (OXTR) is a member of the GPCR family and is 388 a.a. in length (Kimura et al., 1992). The cytoplasmic domain (the C-terminal intracellular domain) is known to functionally couple with q or 11 subtype α subunit (G α q/11) of the trimeric G-protein complex. Activation of OXTR by the binding of OXT to its outer membrane domain sequentially activates G-protein α subunit, phopholipase C and PKC, and finally activates numerous cellular proteins and accelerates the outflow of Ca^{++} from the endoplasmic reticulum, leading to several downstream cellular responses.

OXTR is known to be expressed in mammary gland (Kimura et al., 1998), uterine myometrium (Helmer et al., 1998), adipose precursor cell (unpublished data), cardiac muscle of heart (Gutkowska et al., 1997) and vascular endothelium layer (Thibonnier et al., 1999). The function of OXTR in the contraction of uterine myometrium during parturition, and in the contraction of the smooth muscle layer of mammary alveoli during milk ejection after stimulation by suckling is well known.

More recent studies have demonstrated that centrally released OXT, presumably synthesized in the hypothalamus, acts as a neuromodulator or neurotransmitter through OXTR expressed in specific brain regions, where it affects behaviours, memory and other physiological functions of the central nervous system. For example, a role for OXT in the induction of maternal behaviours has been demonstrated in several animal models in which the administration of OXT facilitates maternal behaviour (Pedersen and Prange, 1979; Williams and Griffith 1992; Nelson and Panksepp, 1998). The receptors for OXT expressed in the central nervous system and in peripheral tissues are considered to be the same molecule.

Generation of oxytocin deficient (*Oxt*−/−) mice

To address the reproductive function of OXT, especially with regard to its role in parturition and milk ejection, we generated OXT gene deficient mice (Nishimori et al., 1996).

Defect in reproductive function in mice deficient in oxytocin gene

Although the resultant *Oxt*−/− mice showed no apparent aberration in physical appearance, growth and reproductive ability, and survived as well as the wild-type mice, newborn pups from *Oxt*−/− dams died shortly after birth due to a disruption in nursing. The histology of the mammary glands of the null postpartum mice showed the accumulation of milk, suggesting that a defect in milk ejection was the most likely cause of the neonatal lethality in their pups (Nishimori et al., 1996). In addition, postpartum intraperitoneal injections of OXT given to the *Oxt*−/− females every few hours enabled sufficient milk ejection to keep several offspring from each litter alive as long as the injections continued (Nishimori et al., 1996).

The indispensable function of OXT for milk ejection was clearly proven by the generation of OXT KO mice. However, contrary to expectations, no defect in parturition by the OXT KO mice was observed; null mice showed quite normal delivery of the newborn pups on the due date. In addition, no gross deficits in maternal nurturing behaviour were detected. These unexpected results raised questions concerning the OXT system. (1) Does the OXTR have a ligand(s) other than OXT that could compensate for the loss in OXT?

(2) Is the OXTR, but not OXT, necessary for normal parturition?

Abnormal social behaviours of **Oxt−/−** *mice*

While *Oxt−/−* displayed grossly normal maternal behaviour, male mutant mice were found to exhibit profound deficits in social recognition, or social amnesia (Ferguson et al., 2000). Interestingly, mutant mice displayed normal cognitive abilities in other tests, such as the Morris Water Maze, and normal olfactory learning for non-social odours. This observation suggests that OXT plays a critical role in the processing of social stimuli, specifically. The deficits in social recognition could be rescued by a central infusion of OXT directly into the amygdala, but not the olfactory bulb (Ferguson et al., 2001).

Generation of oxytocin receptor deficient (*Oxtr−/−*) mice

The lack of the expected phenotype on parturition and maternal behaviour in the *Oxt−/−* mice was particularly puzzling and suggested that alternative ligands or other compensatory effects could have been influencing OXTR activation in the OXT mutant mice. We therefore decided to further define the role of the OXTR system by creating OXTR KO (*Oxtr−/−*) mice (Takayanagi et al., 2005) in collaboration with Dr. Kimura, who first cloned mouse oxytocin receptor gene (Kubota et al., 1996).

Defective phenotype of **Oxtr−/−** *mice in reproductive function*

Oxtr−/− females and males copulate normally, and their fertilization efficiency was also normal. In addition, and surprisingly, the parturition of *Oxtr−/−* pregnant mice was quite normal without any delay compared to that of the WT animals.

On the other hand, as in the case of the phenotype of *Oxt−/−* postpartum mice, *Oxtr−/−* postpartum mice showed a defect in milk let-down, and all neonatal pups born from the *Oxtr* null mother mice died shortly after birth, presumably because of the lack of feeding and resultant dehydration in the pups. Those observations clearly indicated that the OXTR was not necessary for parturition in mice, and that its ligand, OXT, was also not necessary.

We suspected that some substitutive mechanism existed in mice to maintain the normal parturition machinery even when the OXT system was not functioning. To test this hypothesis, we generated mice doubly deficient in the genes for *Oxtr* and prostaglandinF2 α receptor (Fp, Sugimoto et al., 1997), an important myometrium contractile factor. This "double KO" mice still showed almost normal parturition after the administration of progesterone antagonist just before term (manuscript in preparation).

Pharmacological analysis of the role of oxytocin receptor on contractile reaction with vasopressin in myometrial smooth muscle

Using *Oxtr−/−* mice, we pharmacologically analysed the OXTR in mouse uterus, and confirmed that not only OXT, but also arginine vasopressin (AVP) could signal through the OXTR resulting in the induction of contractile movement of myometrial smooth muscle. In contrast, vasopressin receptor Avpr(V1a) is not expressed in mouse uterine myometrium and no signals through Avpr(V1a) are generated to induce contraction in mouse uterine myometrium in vitro.

In the myometrium of non-pregnant mice, both AVP and OXT induced contractions. The effect of OXT was the most potent, while the maximum contractions induced by these two peptides were almost of the same magnitude. Pharmacological characterization of the mouse OXTR, using the *Oxtr−/−* mice as an experimental control, showed that not only OXT but also AVP induced the dose-dependent contraction of uterine myometrium prepared from both non-pregnant and pregnant (Fig. 1A, B) wild-type females. Further experiments showed that both AVP- and OXT-induced contractions were strongly inhibited by an OXTR antagonist, CL-12-42 ($d(CH_2)_5[Tyr(Me)^2,Thr^4,$-$Tyr\text{-}NH_2^9]$OVT; a generous gift from Dr. Maurice Manning, Medical College of Ohio), but weakly

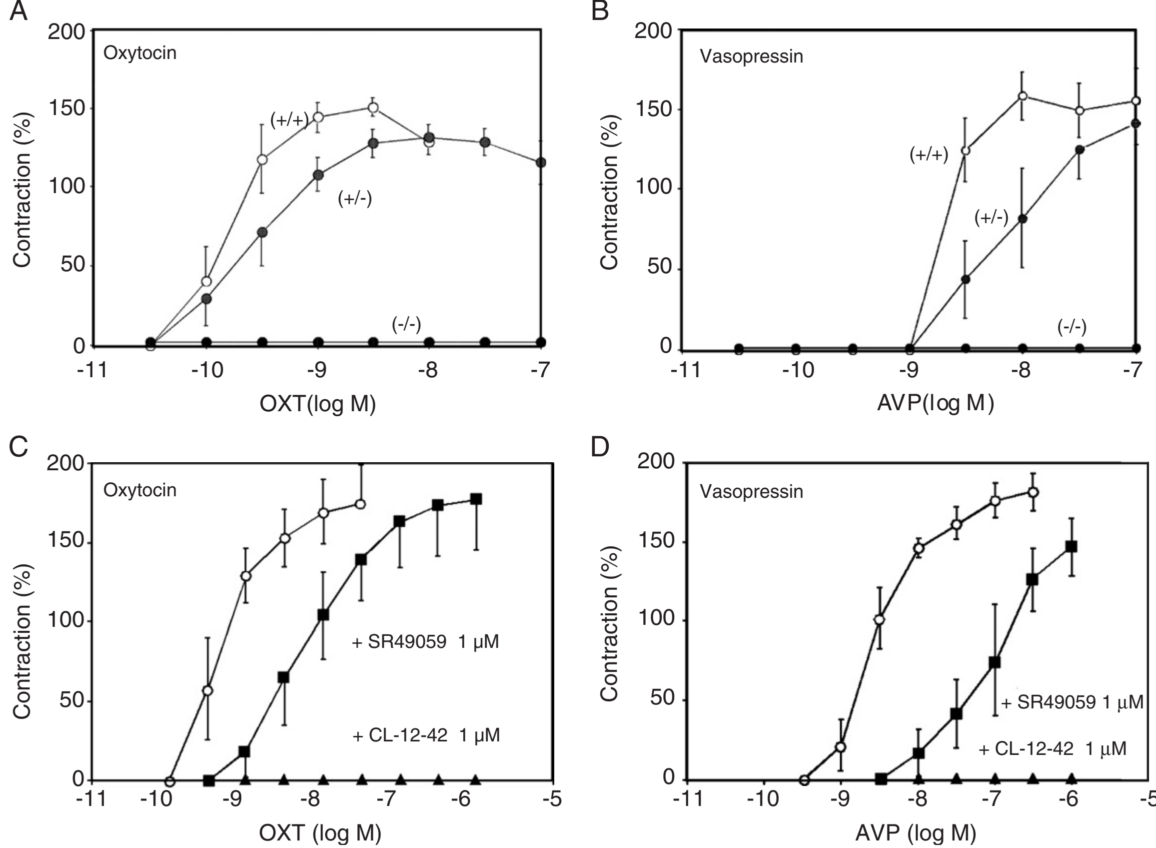

Fig. 1. Cumulative dose–response curve for oxytocin and vasopressin in pregnant mouse myometrium (Adapted with permission from Kawamata et al., 2003). Contractile profiles of myometrium from *Oxtr−/−* pregnant female in response to increased dose of oxytocin (A), or of vasopressin (B) are shown. Contractile profiles of myometrium from WT female in response to increased dose of oxytocin (C), or of vasopressin (D), in the presence of oxytocin receptor antagonist CL-12-42 and V1aRreceptor antagonist SR49059, are shown.

inhibited by a vasopressin Avpr(V1a) antagonist, SR49059; kindly provided by Sanofi Recherche Co. Ltd., France). Similar results were obtained in contraction experiments (Fig. 1C, D) (Kawamata et al., 2003) using myometrium from pregnant WT mice. These results suggested that, in the mouse myometrium, not only OXT- but also AVP-induced contraction was mediated by the activation of OXTRs but not by that of Avpr(V1a) vasopressin receptors. We confirmed the absence of expression of Avpr(V1a) mRNA in the myometrium of WT mice by an RT-PCR procedure (Kawamata et al., 2003). As for human non-pregnant myometrium, in contrast to mouse, we confirmed the inhibition of AVP-induced contraction by SR49059. Our finding, confirmed with experiments using myometrium from *Oxtr−/−* mice, suggests that there are significant differences in the physiological characteristics of OXTRs in contractile responses to AVP and OXT between human and mouse uteri.

Abnormal social behaviours of Oxtr−/− *mice*

Preliminary observations in our *Oxtr−/−* mouse colony suggest an increased frequency of male mice injured in the cages of littermates from *Oxtr+/−* (hetero) parents. We therefore carried out quantitative experiments to measure male aggressiveness using a resident–intruder aggression test.

Elevated aggressive behaviour in Oxtr−/− *male mice*

To test the aggressiveness of the *Oxtr*−/− male mice, we applied a resident–intruder aggression test. Principally, a group-housed C57BL6J male mouse as the intruder (stimulus) was put into the homecage of an individually housed 10-week-old resident male (experimental), and attack duration, frequency and latency to first attack were recorded (see details in Takayanagi et al., 2005). These experiments confirmed increased aggressive behaviour of male *Oxtr*−/− mice (Fig. 2).

Although in our preliminary observation we had not detected a significant elevation in aggressive behaviour in OXT ligand KO mice, we carried out the resident–intruder aggression test to determine the level of aggressive behaviour of *Oxt*−/− male mice as well as that of their wild-type littermates. In contrast to *Oxtr*−/− male mice, *Oxt*−/− male mice did not show any elevation in aggressive behaviour, in comparison with their hetero (*Oxt*+/−) or wild-type littermates. Following this discrepancy between peptide and receptor mutant mice, we hypothesized two explanations: (1) an alternative ligand is activating the OXTR in the *Oxt*−/− knockout or (2) developmental exposure of OXT in the *Oxt*−/− from maternal sources or even siblings.

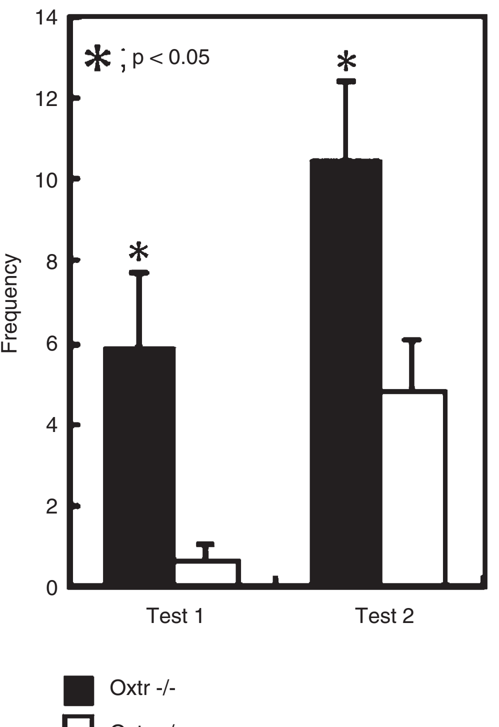

Fig. 2. Aggressive behaviour as measured by the resident–intruder test for *Oxtr*−/− mice (Adapted with permission from Takayanagi et al., 2005). Aggressive behaviour was quantified two times and indicated by attack frequency in the first and second trial (Test 1 and Test 2, 5 min for each test with a 5-min interval. *Oxtr*−/− ($n = 9$) and *Oxt*+/+ ($n = 9$) mice were used.

Supporting the first possibility, Kawamata et al. (2003) reported that AVP, could give an active signal through the OXTR to contract the uterine myometrium in vitro as described in "Pharmacological analysis of the role of oxytocin receptor on contractile reaction with vasopressin in myometrial smooth muscle" of this article. However, AVP apparently failed to suppress aggressive behaviour via the OXTR in *Oxt*−/− mice. We therefore suspected an effect of maternal OXT or OXT synthesized in heterozygous (*Oxt*+/−) or wild-type littermates diffusing through the placental barrier to the null fetus. We normally produced *Oxt*−/− mice by an *Oxt*+/− breeding pair to avoid neonatal lethality of the newborn *Oxt*−/− mice, because of the lack of milk feeding from the null mother mice. This strategy was also good for obtaining all genotypes, such as +/+, +/− and −/−, as littermates, which were ideal for the experiments.

In human, factors whose molecular weight is more than 500 can hardly reach the brain through the blood–brain barrier (BBB), and externally administrated OXT is not considered to be efficiently transported into the brain through this barrier of adult human (Wahl, 2004). However, reports have discussed the possibility of transmission of high molecular hydrophilic materials through the BBB of the fetus, because the foetal barrier may be compromised and the leaked transport of higher molecular weight substances through the BBB of animal embryos may not be negligible (Dziegielewska et al., 1979). In addition, OXT has been reported to be easily transported through placental barrier (Dawood et al., 1979).

Accordingly, we suspected that there was an effect of maternal OXT, diffusing through the placenta and BBB, to the brain of *Oxt*−/− embryos, which might rescue the normal behavioural phenotype including the normally suppressed aggressive behaviours. To test this hypothesis, we generated *Oxt*−/− male mice developed in a maternal environment without possible exposure to OXT by the breeding of *Oxt*−/− parents. Although, in this breeding combination, *Oxt*−/− mothers could not feed their neonatal pups, we cross-fostered them to WT lactating female mice soon after birth to prevent the neonatal lethality of the null pups born from the null breeding pairs. Thus, *Oxt*−/− male mice with the OXTR, but without exposure to OXT during their prenatal development, showed higher aggressive behaviour, similarly to that of *Oxtr*−/− mice (Fig. 3). These results suggested that the aggressive behaviour of *Oxt*−/− male mice, developed in *Oxt*+/− mothers, was suppressed to the normal level by the prenatal exposure of OXTR to maternal plasma OXT, delivered through the premature BBB.

These phenomena suggested the important influence of OXT on prenatal brain development in uteri by which normal social behaviours are established later in adult mice. This may be one of the typical molecular mechanisms by which the maternal milieu regulates foetal development and determines behavioural features in adulthood. In addition, at least in the case of mice, our data showed that appropriate level of exposure of the embryonic brain to OXT was very important for regulating the aggressive response in adulthood.

Further, for some human autistic disorders, a contribution of OXT administered to the pregnant mothers for labour induction has been suggested (Gale et al., 2003). Our observations concerning this suggested mechanism by which aggressive behaviour was established in adult *Oxtr*−/− and

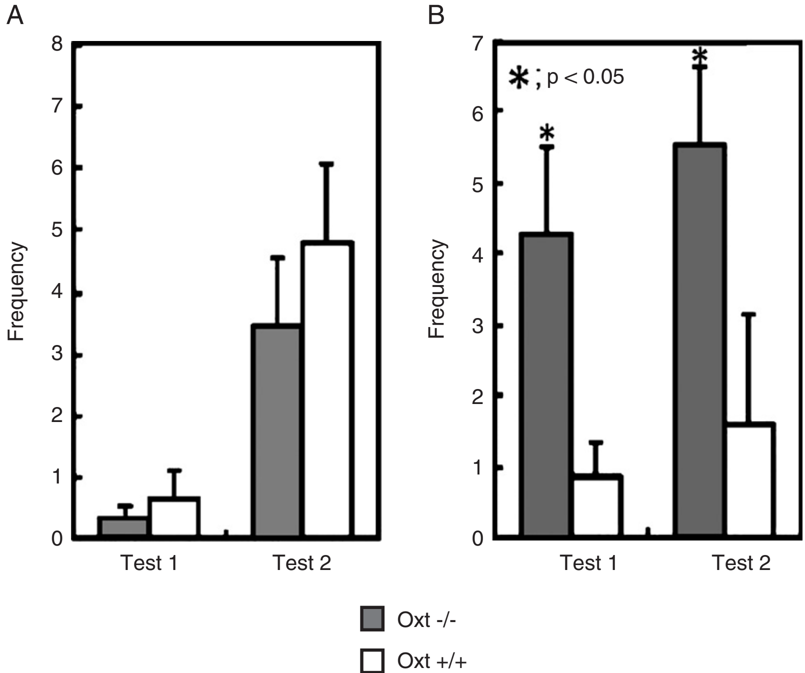

Fig. 3. Aggressive behaviour by the resident–intruder test for *Oxt*−/− mice (Adapted with permission from Takayanagi et al., 2005). Aggressive behaviour was quantified two times and indicated as in Fig. 2. *Oxt*−/− ($n = 11$) and *Oxt*+/+ ($n = 9$) mice from heterozygous intercrosses (A), or *Oxt*−/− ($n = 8$) and *Oxt*+/+ ($n = 7$) mice from intercrosses of homozygous *Oxt*−/− and *Oxt*−/− mice fostered by C57BL/6J females (B), were used. $^{*}p = 0.05$ (Mann–Whitney U test). Error bars indicate standard error.

Oxt−/− mice could contribute to understanding some of these disorders. As described above, we first observed increased aggressive behaviour in *Oxtr*−/− male mice. Next, we studied differences in other social behaviours, such as social recognition, maternal behaviours and isolation induced pup ultrasonic vocalizations, because of increasing reports suggesting the importance of neurohypophysial hormones and their receptors in animal social behaviours.

Maternal behaviours of Oxtr−/− *female mice*

We then examined retrieving behaviour and crouching over pups to determine whether aberrations in the maternal behaviours were present in *Oxtr*−/− female mice. Maternal behaviour was assessed in both postpartum (13–18 weeks old) and virgin females (7–9 weeks old) (Fig. 4). Maternal behaviour disruptions were present in both postpartum and virgin *Oxtr*−/− mutant female mice. This observation is in contrast with our previous finding of no gross disruption in maternal behaviour in OXT ligand KO mice. In these experiments, unaffected maternal behaviours were observed in *Oxt*−/− female mice, even those derived from breeding pairs of *Oxt*−/− parents followed by adoption of the newborn *Oxt*−/− mice to the wild-type foster mothers. These observations suggest that the establishment of maternal behaviours in mice requires the presence of OXTR, regardless of the existence of Oxt gene. It is possible, in this case, that other ligands may provide sufficient OXTR activation in the *Oxt*−/− to mediate the development maternal responsiveness.

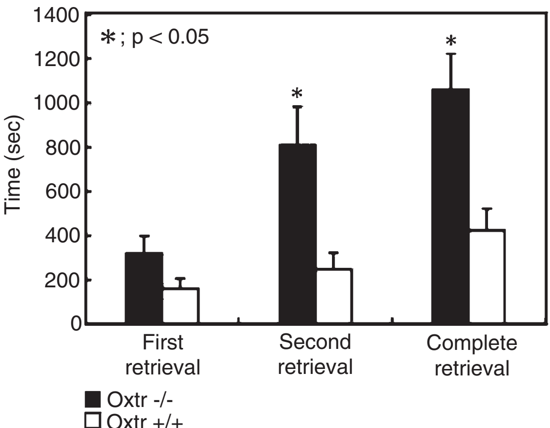

Fig. 4. Maternal nurturing behaviour in female *Oxtr*−/− mice. (Adapted with permission from Takayanagi et al., 2005). Pup retrieval behaviours were tested with *Oxtr*−/− ($n = 9$) or *Oxtr*+/+ ($n = 10$) females from heterozygous intercrosses. Failure to retrieve was assigned as 30 min, the length of the observation period. $^{*}p = 0.05$ and $^{**}p = 0.01$ (Mann–Whitney *U* test). Error bars, standard error.

Social recognition

As mentioned previously, *Oxt*−/− male mice displayed a social amnesia phenotype (Ferguson et al., 2000), strongly suggesting a relationship between the of OXT-receptor system and the control of social behaviour such as social discrimination. A social discrimination test (Landgraf et al., 2003) was also done for *Oxtr*−/− male mice (4–7 months old), singly housed in their own homecage into which group-housed ovariectomized mice were introduced (Ferguson et al., 2000; Takayanagi et al., 2005). We also confirmed that *Oxtr*−/− mice had a defect in social recognition ability as did *Oxt*−/− male mice.

Mother–offspring interaction

Ultrasonic vocalizations (USV) in rodents are signals that play an important communicative role in mother–offspring interaction, and USV of an infant and even those of adult rodents are known to be altered in tone and frequency in response to their emotional situation, suggesting that USV could be used as a quantitative measure of the emotional state such as fear and anxiety in animal models. To understand the effect of a deficit of OXTR on mother–offspring interaction, we examined the isolation-induced USV of *Oxtr*−/− male infants at postnatal day (P)7 when they were separated from their mother. *Oxtr*−/− pups displayed significantly fewer calls than their wild-type littermates (Fig. 5), as *Oxt*−/− males also did (Winslow et al., 2000), while displaying significantly higher levels of locomotor activity during

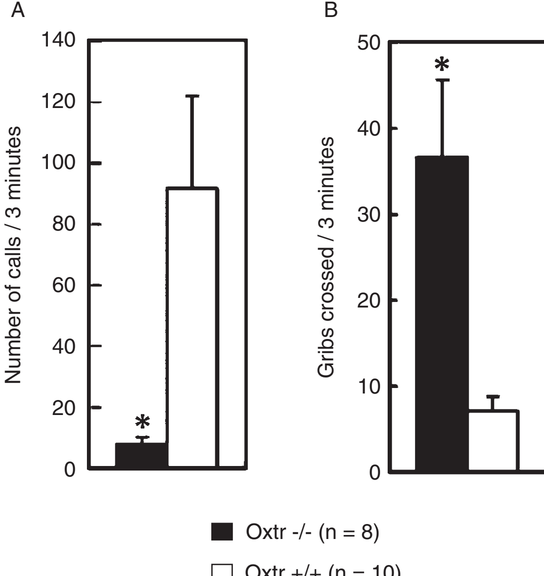

Fig. 5. Infant ultrasonic vocalization (Adapted with permission from Takayanagi et al., 2005). (A) Ultrasonic vocalization of 7-day-old *Oxtr*−/− male pups from Oxtr hetero breeding pairs was tested as described (Takayanagi et al., 2005). The parents were removed from the home cage 20 min before testing. Vocalizations were recorded by using an ultrasonic detector and analysed. (B) Locomotor activity of the same pups.

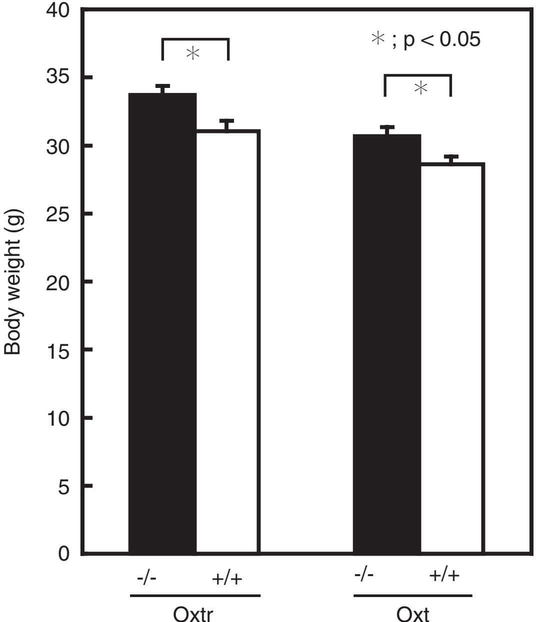

Fig. 6. Body weight of WT ($n = 13$) and *Oxt*−/− ($n = 18$) male mice. *$p < 0.05$ compared with WT mice (20-week-old).

the test. These results from *Oxtr*−/− male pups, showing less USV and more locomotor activity, suggested that the pups without OXTR were more resistant against social isolation and might shift their behaviour towards more exploratory activity (Takayanagi et al., 2008).

Function of oxytocin in energy balance and control of body temperature homoeostasis

The *Oxtr*−/− mice that we generated showed several abnormal phenotypes other than defects in reproductive function and sociosexual behaviours. For example, the *Oxtr*−/− male mice showed slight obesity with aging, and especially 12 weeks after birth or later, they showed a remarkable increase of body weight together with an increase of white adipose tissue (WAT) (Fig. 6) (Takayanagi et al., 2008).

However, they did not show a difference in food intake as compared with the wild-type mice. On the other hand, especially their visceral fat weight increased and enlargement of adipocytes with the accumulation of lipid was observed in both WAT and brown adipose tissue (BAT). In addition, BAT, known as a major tissue generating heat in rodents, showed cells apparently accumulating excessive lipids inside. Because several types of gene-KO mice with abnormal brown adipocytes show aberrations in the generation of heat (Cannon and Nedergaard, 2004), we hypothesized that *Oxtr*−/− mice would also show deficits in heat generation when exposed to cold (5°C). To test this hypothesis, we measured rectal temperature periodically for 2 h during exposure to cold. This test revealed that rectal temperature was significantly decreased in *Oxtr*−/− mice in comparison to that of WT mice (Fig. 7). These tendencies of slight obesity in adult male mice and a rapid decrease of body temperature after exposure to cold were also observed with *Oxt*−/− mice (Kasahara et al., 2008).

We carried out additional experiments to study the mechanism that caused the obesity and aberration in body temperature control observed

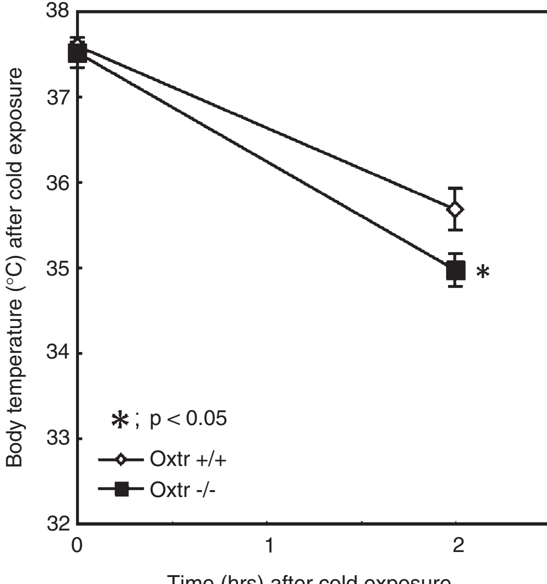

Fig. 7. Body temperature shifts of wild-type (WT, *Oxtr*+/+) and *Oxtr*−/− mice before and after cold exposure. The rectal temperature in 10–12-week-old male WT ($n = 9$) and *Oxtr*−/− ($n = 10$) mice is shown. The initial time point (point 0) shows the measured temperature after 3 h fasting and just before cold exposure (at 5°C). Data shown were mean ± SEM. *Significant difference between WT and *Oxtr*−/− mice.

in both *Oxt*−/− and *Oxtr*−/− mice. Noradrenalin (NA) released from sympathetic nerve terminals regulates heat generation in BAT through adrenergic receptors. We analysed the expression of adrenergic receptors in the BAT of *Oxtr*−/− mice. Adrenergic receptors (AR) are classified into several subtypes, including β3-AR, which accelerates heat generation and lipid oxidation, and α2A-AR, which suppresses lipid catabolism. Quantitative RT-PCR for both ARs mRNA in the BAT of *Oxtr*−/− male mice showed increased expression of α2A-AR and remarkable suppression in the expression of β3-AR indicating a declination in the balance of energy expenditure might have occurred in the *Oxtr*−/− mice to save energy (Kasahara et al., manuscript in preparation). In addition to reports that receptor activity for OXT (Boland and Goren, 1987) or OXTR mRNA was detected (Gould and Zingg, 2003) in adipose tissue, we observed OXTR mRNA mainly expressed in adipocyte precursor cells (unpublished data) but the expression level of the receptor mRNA in mature adipocyte was quite low. All of these findings suggest that the lack of OXTR in adipose precursor cells and the resultant dysfunction in mature adipose tissue may not be the major cause of obesity in *Oxtr*−/− (and even in *Oxt*−/−) mice. On the other hand, the hypothalamus, the center of neurons expressing OXT, is also the center that controls the body temperature. We detected co-localization of OXT and c-fos protein by immunostaining in a portion of neurons in the hypothalamus (PVN) after exposure of WT mice to 5°C for 2 h (Kasahara et al., 2008). These observations strongly suggested that the OXT–OXTR system in the central nervous system contributes to temperature homoeostasis and presumably to the resultant energy balance in the body. With the hypothesis that the imbalance in energy uptake versus intake, presumably caused by the deficit of OXT or OXTR genes in the mice, might give rise to obesity and aberrations in body temperature control after cold exposure, we are further analysing the mechanism causing these phenotypes.

Generation of Oxtr-Venus knockin mice to locate "Oxtr-Neurons"

Although the cloning of the OXTR was achieved more than a decade ago, an effective and reliable anti-OXTR antibody to locate the neurons that express OXTR, to identify the subtype of the neurons, or to delineate circuits including OXTR-neurons has not been produced. Recently, to obtain tools to identify the regions where several important genes are expressed in the brain, the GENSAT project (URL; http://www.ncbi.nlm.nih.gov/projects/gensat) generated several transgenic mice lines expressing EGFP under the control of those genes. The GENSAT project was based on the generation of transgenic mice using BAC clone-derived genomic DNA to control the expression of the marker EGFP gene. This project is of great interest for researchers because in each of the bacterial artificial chromosomes (BAC) transgenic vectors, each of the endogenous protein coding sequences have been replaced by sequences

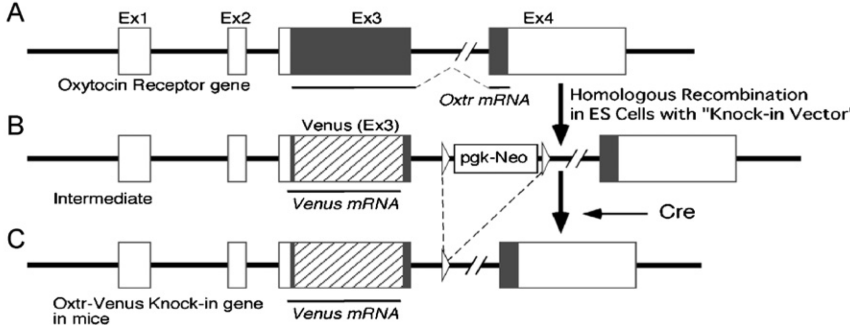

Fig. 8. Strategy for generation of *Oxtr-Venus* knockin mice. (A) Gross intron–exon structure of Oxtr gene in a wild-type mouse. (B) Primary structure of mouse Oxtr gene locus after generation of floxed type Oxtr-Venus knockin mouse using ES cell technology. (C) Final structure of Oxtr gene locus of Oxtr-Venus knockin mouse after deletion of LoxP-Neo-LoxP cassette by crossing with CAG-Cre mouse.

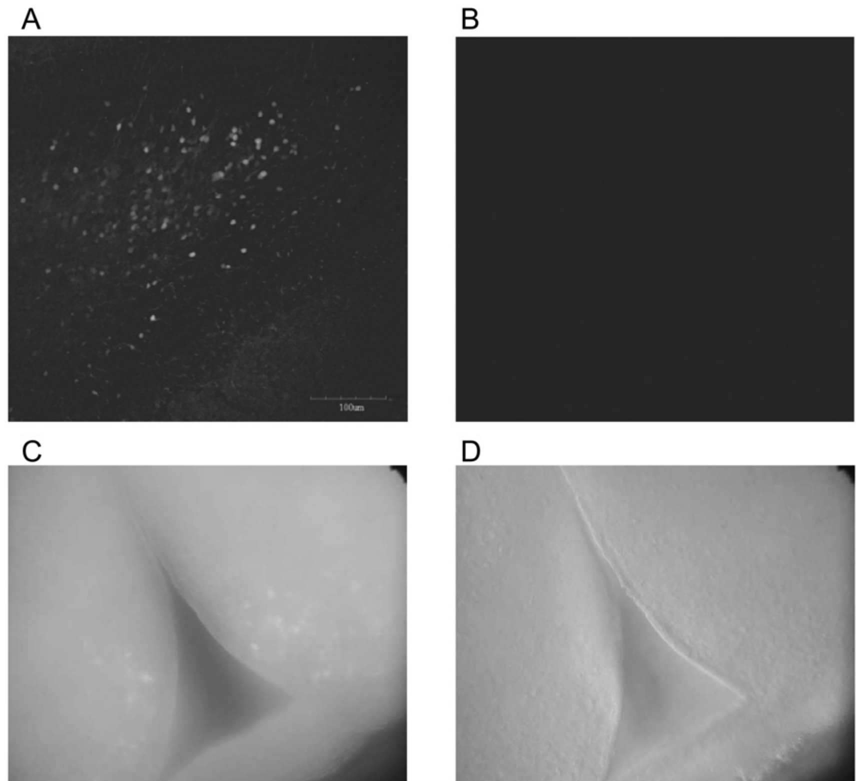

Fig. 9. Venus fluorescence of *Oxtr-Venus* knockin mice. (A, B) Brain sample of *Oxtr-Venus* knockin mice (P40), or control WT mouse (P40) was observed by confocul laser scanning microscope after fixation by PFA perfusion, preparation of sections by cryostat and staining with Anti-EGFP Ab. The original fluorescence of the Venus protein was enhanced by staining with FITC-conjugated secondary antibody. Pictures show neurons emitting fluorescence at the olfactory bulb. (C) Slice of brain (live organ culture) sample prepared from Oxtr-Venus knockin mice (P40) observed by fluorescence microscope. At the arcuate region, many neurons showed clear fluorescence suggesting that it is suitable for further electrophysiological analysis. (D) The same slice under a visible field.

encoding the EGFP reporter gene making them easily detectable and measurable. However, using this strategy one must carefully consider whether the distribution of the EGFP marker is equal to that of the endogenous gene products, because the expression of multiply inserted trans-genes may be affected by the circumstances of the chromosomal locus where they are randomly integrated.

Recently, we independently generated *Oxtr-Venus* knockin mice, whose OXTR coding region in the chromosome was substituted by the Venus (enhanced YFP, Nagai et al., 2002)-coding sequence, using homologous recombination. The aim of generating *Oxtr-Venus* knockin mice is to obtain a tool to characterize Oxtr-producing cells, and to identify live Oxtr-neurons for various subsequent analyses such as electrophysiological experiments, with minimal interference that might be caused by artificial manipulation of the gene responsible for the intrinsic expression of OXTR.

The *Oxtr-Venus* knockin vector (Fig. 8) was composed of the OXTR gene sequence, whose exon 3 was substituted with the Venus structural gene sequence, and was introduced into embryonic stem (ES) cells using a conventional technique. With the resultant *Oxtr-Venus* knockin mice, we detected Venus fluorescence in the lateral septum (LS), cortical amygdaloidal nucleus, medial amygdaloidal nucleus, arcuate nucleus, olfactory nucleus and so on (Fig. 9) (Yoshida et al., submitted). The *Oxtr-Venus* knockin mouse may be an ideal experimental tool for further analysis such as electrophysiology that require accurate information concerning the distribution of the Oxtr-neurons and identification of their subtype.

Abbreviations

a.a.	amino acids
AVP	arginine vasopressin
AVPR(V1a)	vasopressin receptor V1a
BBB	blood brain barrier
ES	embryonic stem
GPCR	G-protein coupled receptor
OXT	oxytocin
OXTR	oxytocin receptor
USV	ultra sonic vocalization
WT	wild type

Acknowledgements

We thank T. Onaka (Jichi Medical School, Tochigi, Japan), T. Kimura (Osaka University, Osaka Japan), K. Itoi (Tohoku University, Miyagi, Japan) for collaborations, technical assistance, discussion and encouragement. This work was supported in part by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (14360046) and by the Sankyo Foundation of Life Science. M.Y. was supported by a Japan Society for the Promotion of Science Research Fellowship for Young Scientists.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 9

Regulation of vasopressin release by co-released neurotransmitters: mechanisms of purinergic and adrenergic synergism

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Abstract: Arginine vasopressin (AVP) neurons of the hypothalamo-neurohypophseal system (HNS) are innervated by numerous afferent pathways carrying information about two physiologically important parameters: blood volume/pressure and osmolality. These pathways use a variety of neurotransmitters/neuropeptides. In order to understand normal and pathological regulation of VP secretion, the mechanisms underlying integration of these complex afferent signals by the AVP neurons must be understood. The importance of neurotransmitter interactions in determining hormone release is highlighted by the finding that simultaneous exposure to adenosine triphosphate (ATP, a neurotransmitter acting on purinergic receptors) and phenylephrine (PE; to mimic norepinephrine activation of α1-adrenergic receptors) results in potentiation of AVP release that is characterized by an increase in the peak response and conversion of a transient response to a response that is sustained for hours. Evaluation of the mechanisms responsible for this response indicated that (1) activation of P2X purinergic receptors (P2X-R) is required, (2) protein kinase C (PKC) activation is required, (3) the sustained component requires new gene transcription, (4) the synergism does not involve presynaptic mechanisms nor does it occur directly in the neural lobe and (5) live-cell Ca^{++} imaging techniques demonstrated a sustained increase in $[Ca^{++}]_i$ and that ATP activates P2Y-Rs as well as P2X-Rs in supraoptic neurons. Since the subtypes of P2X-Rs differ in their rate of desensitization, identification of the subtype of P2X-Rs participating in the initial and sustained responses to ATP + PE may elucidate mechanisms underlying the abrupt and transient responses to orthostatic hypotension versus sustained responses to chronic hypovolemia or vasodilation.

Keywords: vasopressin; oxytocin; ATP; purinergic; norepinephrine; adrenergic; haemorrhage; shock

Introduction

The hormones secreted from the hypothalamo-neurohypophyseal system (HNS) regulate important homeostatic functions. Arginine vasopressin (AVP) is critical for maintenance of water balance and cardiovascular function via its antidiuretic and vasoconstrictor actions. Oxytocin (OTX), in addition to its roles in reproduction (lactation and parturition), also participates in maintenance of fluid and electrolyte balance via its natriuretic effects (Verbalis et al., 1991). Sustained elevations in AVP and OTX

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DOI: 10.1016/S0079-6123(08)00409-3

secretion persist for days in response to dehydration (Dunn et al., 1973; Windle et al., 1993) which, as a combined osmotic and hypovolemic stimulus, activates CNS pathways carrying information about plasma osmolality as well as the pathways carrying information about blood pressure and volume. Prolonged decreases in blood pressure and blood volume independent of hyperosmolarity also induce elevations in plasma AVP and OTX that can be sustained for hours to days. Hypotension induced by intravenous injection of hydralazine, a potent vasodilator, induces large increases in both plasma OTX and AVP for at least 90 min (Schiltz et al., 1997). Both non-hypotensive and moderate (20% of blood volume) haemorrhages induce prompt and prominent increases in plasma AVP (Grimes et al., 1987; Block et al., 1989; Blair et al., 1991). Without intervention, blood volume restitution in response to a moderate haemorrhage can require more than 24 h (Grimes et al., 1987; Blair and Mickelsen, 2006). Cardiovascular collapse occurring in haemorrhagic and septic shock and in patients suffering from cardiac arrest or following extended cardiopulmonary bypass is associated with a failure of AVP secretion to maintain plasma AVP at levels sufficient to prevent vasodilation and can be averted by administration of exogenous AVP (Morales et al., 1999; Landry and Oliver, 2001; Sharshar et al., 2003). Thus, sustained elevation in AVP secretion in response to hypotension and hypovolemia is critical for maintaining cardiovascular homeostasis.

Pathways transmitting hypotension and hypovolemia signals to AVP neurons

As previously reviewed (Sladek, 2000), AVP secretion in response to cardiovascular information is transmitted to the AVP neurons by multiple pathways carrying information about decreases in blood pressure and blood volume and a separate inhibitory pathway carrying information about increases in blood pressure. Baroreceptors in the carotid sinus and stretch receptors in the right atria of the heart monitor blood pressure and volume, respectively. This information is carried to the nucleus tractus solitarius and dorsal vagal complex in the brainstem by the IXth and Xth cranial nerves, and the information is relayed to the A1 and C1 catecholamine neurons in the ventrolateral medulla which in turn innervate the AVP neurons of the HNS. Destruction of either the A1 (noradrenergic) projection to the supraoptic nucleus (SON) or the C1 (epinephrinergic) neurons of the rostral ventrolateral medulla compromises the response of AVP neurons to hypotension or simulated hypovolemia (Day and Sibbald, 1990; Smith et al., 1995; Madden et al., 2006).

Neurotransmitters responsible for stimulation of AVP neurons in response to hypovolemia/hypotension

Although the A1 pathway was first recognized for its ability to secrete norepinephrine (NE) and convincing evidence supported the importance of the A1 pathway for stimulation of AVP release in response to moderate decreases in blood pressure (Raby and Renaud, 1989; Smith et al., 1995), the studies that intended to demonstrate that NE is the transmitter responsible for this response were unsuccessful (Day et al., 1990). Adrenoceptor antagonists did not block A1 activation of AVP cells, prompting the suggestion that these neurons use neurotransmitters in addition to NE as their principal transmitter (Day et al., 1990). Injections of the broad spectrum excitatory amino acid (EAA) receptor antagonist, kynurenic acid, were ineffective in blocking excitation induced by stimulation of the A1 region, ruling out the possibility that glutamate is responsible for AVP responses to A1 input (Day et al., 1990). Neuropeptides such as neuropeptide Y and substance P that are co-localized in subsets of A1 neurons (Everitt et al., 1984; Sawchenko et al., 1985; Blessing et al., 1986; Bittencourt et al., 1991) were considered candidates for transmitting cardiovascular information, but were found to play modulatory roles (Willoughby and Blessing, 1987; Sibbald et al., 1989; Kapoor and Sladek, 2001). A role for ATP, the nucleotide which is commonly co-localized in catecholamine vesicles (Fried, 1980; Whittaker, 1982), in mediating responses to activation of the A1 pathway was supported by the finding that application of the P2 receptor blocker, suramin (10 mM), in the SON reversibly blocked the excitation of AVP cells by A1

stimulation without preventing the excitatory effect of locally applied NE (Day et al., 1993). Histological and electrophysiological evidence that SON neurons express multiple subtypes of purinergic receptors, the receptor family activated by ATP, further supports a role for purinergic transmission (Hiruma and Bourque, 1995; Shibuya et al., 1999) and supports a role for ATP as a neurotransmitter involved in cardiovascular regulation of AVP secretion.

Response to ATP and activation of α1-adrenergic receptors

The above evidence as well as data demonstrating that ATP and NE are co-released in hypothalamic slices (Sperlagh et al., 1998) prompted us to evaluate the impact of co-exposure to ATP and NE on AVP and OTX release. Since our early experiments had demonstrated that the effect of NE on AVP release was situation dependent due to offsetting effects on α- and β-adrenergic receptors (Sladek and Yagil, 1990), we examined whether the response to ATP and phenylephrine (PE), an α1-adrenergic receptor (α1-R) agonist that excites SON neurons and stimulates AVP release (Armstrong et al., 1986), was different when the agents were applied separately or together. As shown in Fig. 1, using acutely prepared and perifused explants of the HNS that include the SON neurons with their axons projecting through the median eminence and terminating in the neural lobe, we observed that the combined exposure to ATP and PE (ATP + PE) resulted in a significantly larger increase in AVP release than was observed with either agent alone and even more impressively, the response was converted from a transient increase in AVP release to one which was sustained for several hours (Kapoor and Sladek, 2000). There was a similar synergistic effect of ATP + PE on OTX release. As is more evident in Fig. 2B, the synergistic response to ATP + PE is frequently characterized by an initial, rather small transient increase in hormone release followed by a delayed, larger increase in hormone release. We have invested considerable effort in understanding the cellular and molecular mechanisms underlying the sustained increase in hormone release, because this

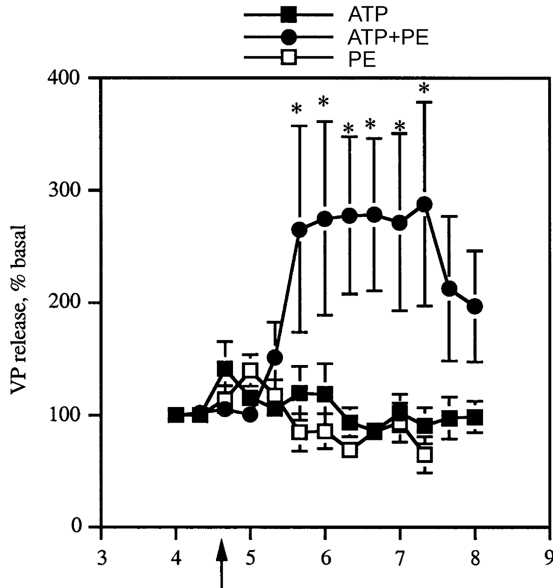


Fig. 1. Effect of ATP (100 μM), PE (100 μM) and combined exposure to ATP + PE (100 μM each) on AVP release from HNS explants. Both ATP and PE alone caused a significant but transient increase in AVP release, but that response is dwarfed by the response to the two agents together. The augmented response to ATP + PE occurs following a delay of approximately 40 min and is sustained for several hours. Adapted with permission from Kapoor and Sladek (2000).

may be critical for maintenance of cardiovascular function during haemorrhage or chronic hypotensive conditions induced by cardiac failure or arrest, vasodilation or cardiopulmonary bypass surgery.

Mechanism of ATP + PE synergism

Presynaptic vs. postsynaptic site of action of ATP and PE

Since SON neurons express both purinergic and adrenergic receptors, it is tempting to assume that ATP and PE act postsynaptically to alter AVP secretion. However, it is also possible that one of these agents acts presynaptically to increase excitatory afferent input (i.e. glutamate release). ATP has been shown to stimulate the release of glutamate and GABA in SON (Ponzio and Hatton, 2004), and NE increases excitatory postsynaptic

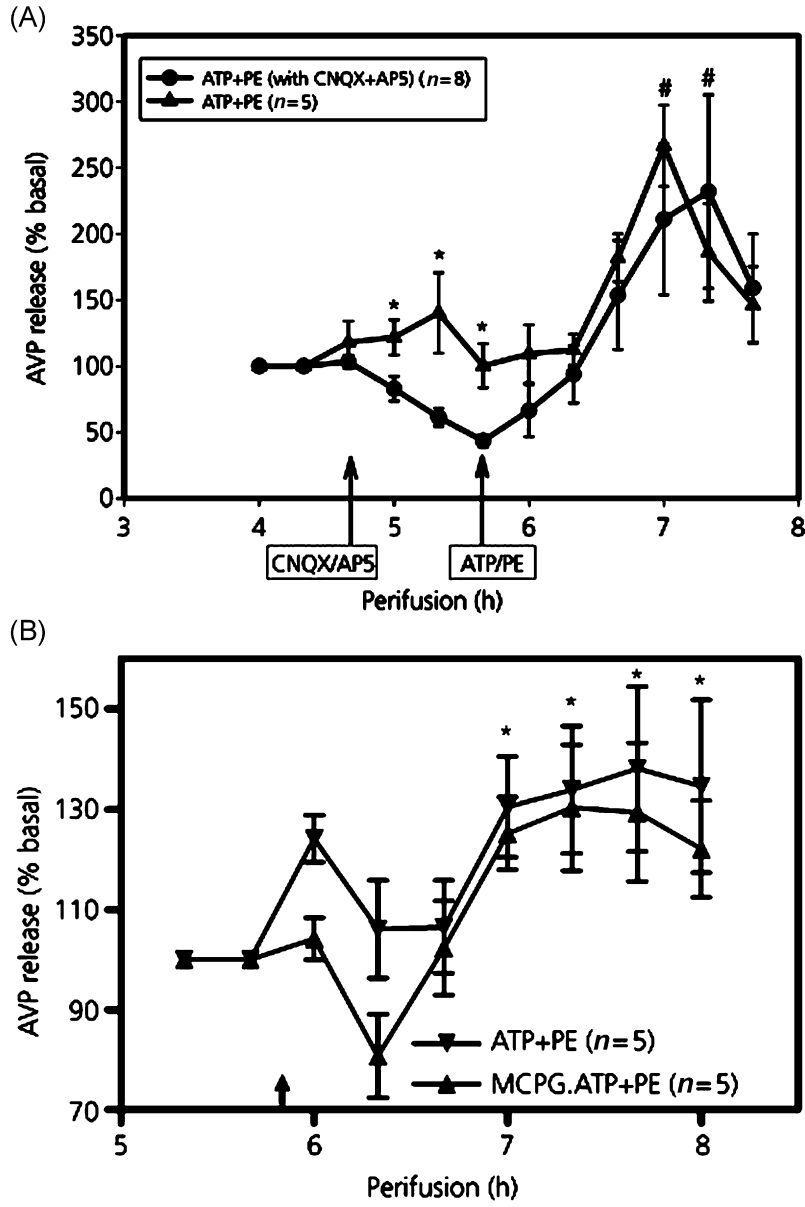


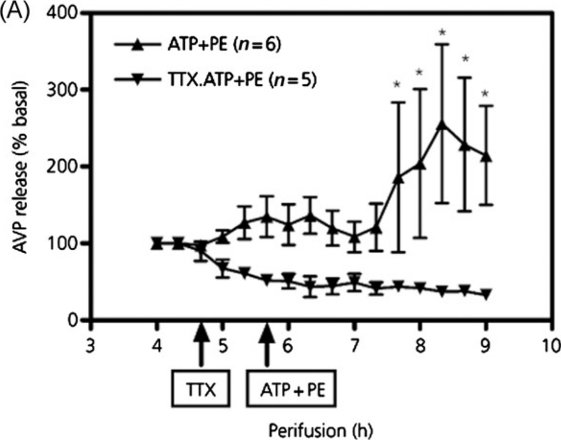
Fig. 2. Effect of excitatory amino acid receptor antagonists on sustained response to ATP + PE. Neither combined exposure to AMPA and NMDA antagonists (CNQX and AP5 respectively, A) nor an antagonist of metabotropic glutamate receptors (MCPG, (RS)-a-methyl-4-carboxyphenylglycine, B) prevented the sustained response to ATP + PE. Adapted with permission from Song and Sladek (2006).

potentials (epsp) on magnocellular neurons via an α1-R-mediated effect (Daftary et al., 1998; Gordon and Bains, 2005). This suggests noradrenergic regulation of local glutamatergic neurons that are known to exist in the perinuclear zone of SON. The possibility of presynaptic actions of ATP and/or NE was addressed using a cocktail of EAA receptor antagonists. Combined exposure to CNQX and AP5 (antagonists at AMPA and NMDA receptors, respectively) decreased basal AVP release but

did not alter the response to combined exposure to ATP and PE (Fig. 2A; Song and Sladek, 2006). The decrease in basal release indicates that glutamatergic tone contributes to basal AVP release in HNS explants, but the synergistic action of ATP + PE is not dependent on activation of ionotropic EAA receptors. A non-selective metabotropic glutamate receptor (mGluR) antagonist [MCPG, (RS)-a-methyl-4-carboxyphenylglycine], also did not alter the response to ATP + PE (Fig. 2B; Song and Sladek, 2006). This does not eliminate the possibility that either ATP or PE acts presynaptically to alter the release of some other excitatory input; however, glutamate is the predominant local excitatory input to SON (Van den Pol et al., 1990) and, therefore, the prime candidate for presynaptic modulation in HNS explants.

Do ATP and/or PE act on the neurohypophyseal nerve terminals?

Purinergic and α1-R mediated effects have also been reported in the neural lobe. ATP has been reported to increase AVP release from isolated neurohypophyseal terminals (Troadec et al., 1998), and to alter K^+ efflux from pituicytes (Troadec et al., 2000). α1-Rs are present in the neural lobe (DeSouza and Kuyatt, 1987), and it receives noradrenergic innervation from the A2 neurons in the medulla as well as sympathetic innervation from the superior cervical ganglion (Alper et al., 1980; Saavedra, 1985; Garten et al., 1989). The possibility that the nerve terminals in posterior pituitary are the site of synergism was addressed by perifusing isolated neural lobes with ATP and PE alone or together. Synergistic stimulation of AVP release by ATP + PE was not observed in isolated, perifused neural lobes (Fig. 3; Song and Sladek, 2006). Furthermore, as shown in Fig. 3, blocking action potential propagation with tetrodotoxin (TTX, 3 μM) eliminated the response to ATP and PE indicating that action potentials are required for the response to ATP + PE (Song and Sladek, 2006). Note that TTX also decreased basal release indicating that a large portion of the hormone release from HNS explants reflects action potential initiated exocytosis. Thus, the synergistic



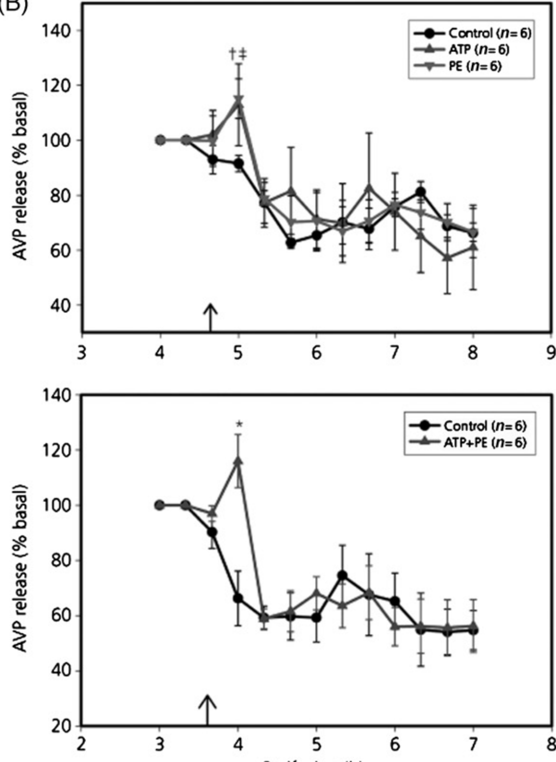


Fig. 3. (A) Tetrodotoxin (TTX) inhibition of action potential generation prevents ATP + PE-induced AVP release from HNS explants. (B) Synergistic stimulation of AVP release is not observed when only the nerve terminals in the neural lobe are exposed to ATP + PE. The small transient response observed reflects the previously reported ability of ATP to activate purinergic receptors in isolated neurohypophyseal terminals (Troadec et al., 1998) and the evidence that noradrenergic innervation of the neural lobe contributes to AVP release in response to hypotension (Stocker et al., 2006). Adapted with permission from Song and Sladek (2006).

effect is not due to synergistic actions of P2 and α1-Rs occurring at the level of the neural lobe, and it requires action potentials initiated in either the hypothalamus or the neural lobe.

Role of adenosine to the ATP+PE synergistic stimulation of AVP/OTX release

ATP is metabolized to adenosine in the extracellular space by ecto-5′-nucleotidase (Dunwiddie et al., 1997). Adenosine inhibits SON neurons (Ponzio and Hatton, 2005), and adenosine receptors (AR) are expressed in SON neurons (Noguchi and Yamashita, 2000). Since conversion of a transient ATP-induced response to sustained stimulation of AVP and OTX release is an important feature of the ATP + PE synergism, removing an inhibitory influence might underlie the development of synergism. Since adenosine is a metabolite of ATP and is inhibitory to SON neurons, we postulated that metabolism of ATP to adenosine could be responsible for the transient response to ATP alone. This possibility was tested using a combination of a potent ecto-5′-nucleotidase inhibitor, α,β-methylene adenosine 5′-diphosphate (AMP-CP), and a competitive substrate for ecto-5′-nucleotidase (guanosine monophosphate, GMP). Enzymatic inhibition did not affect basal AVP release. It did slightly prolong the response to ATP, but not for the duration of exposure to ATP; the response to ATP was not greater than that observed with ATP alone (Fig. 4; Song and Sladek, 2005). Therefore, although conversion of exogenously applied ATP to adenosine contributes to termination of ATP-induced stimulation of AVP release, production of adenosine alone cannot account for the sustained responses observed with ATP + PE.

Purinergic receptor subtypes activated by ATP in SON neurons

Extracellular ATP acts as a neurotransmitter by activation of two classes of purinergic receptors: P2X receptors (P2X-R) and P2Y receptors (P2Y-R).

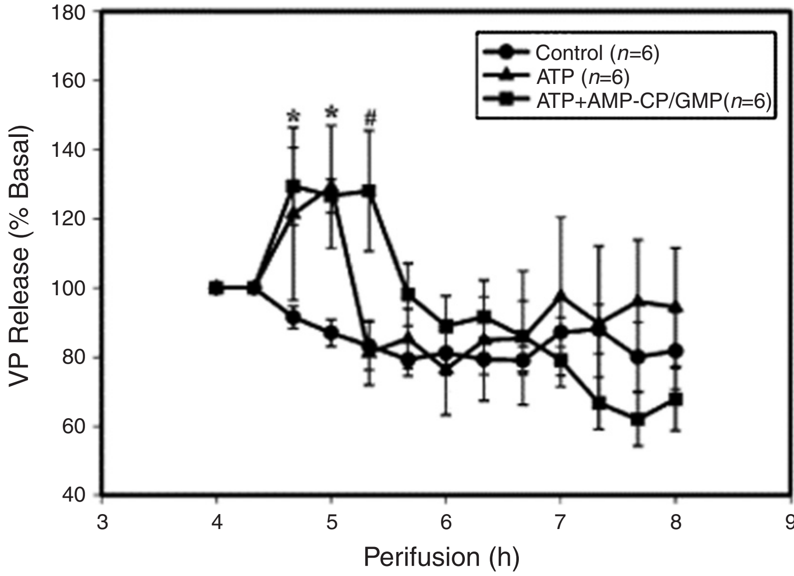


Fig. 4. Effect of enzymatic blockade of ATP metabolism on ATP-induced AVP release. The cocktail of α,β methylene adenosine 5′ diphosphate (AMP-CP), a potent ecto-5′-nucleotidase inhibitor, and guanosine monophosphate (GMP), a competitive substrate of ecto-5′-nucleotidase, provided a short extension of the AVP response to ATP, but did not convert it to a response that was sustained for the duration of exposure to ATP as occurs with simultaneous exposure to ATP + PE. This supports the conclusion that metabolism of ATP to adenosine is not responsible for the transient AVP response induced by ATP. Adapted with permission from Song and Sladek (2005).

P2X-R subtypes

The P2X-Rs are ligand-gated non-selective cation channels that allow Na^+ and Ca^{++} to enter the cell. There are seven subtypes that are coded by different genes (P2X1-7-R). P2X-Rs are composed of three subunits, and heteromeric receptors can be formed by combinations of subunits of different receptor subtypes. The P2X-R subtypes differ in their rate of ligand-induced desensitization, some demonstrate pore-forming characteristics, and to some extent they can be differentiated by their sensitivity to agonists and antagonists (see Table 1; North, 2002). mRNA for all of the subtypes except P2X1 and 5-R has been detected in SON using in situ hybridization or RT-PCR with P2X3, 4 and 7-R predominant (Collo et al., 1996; Shibuya et al., 1999). Although prominent immunoreactivity for P2X5-R was recently reported in SON and PVN (Xiang et al., 2006), the functional contribution of this subtype remains speculative due to the small amplitude of ATP-induced currents via this receptor and the apparent absence of mRNA in SON. Functional evidence for expression of P2X3-Rs (or P2X2/3-Rs) in SON was provided by the ability of the selective agonists, α,β-methylene-ATP and 2-methylthio-ATP, to mimic the depolarizing effects of ATP on SON neurons (Hiruma and Bourque, 1995). The ability of 10 μM PPADS (pyridoxal-phosphate 6-azophenyl 2′,4′-disulphonic acid) to block the synergistic effect of ATP + PE on AVP release (Fig. 5; Kapoor and Sladek, 2000) is consistent with this effect requiring activation of P2X2-R, P2X3-R and/or P2X2/3-Rs, because the other candidate P2X-Rs are relatively insensitive to PPADS (Table 1). Similarly, involvement of P2X2, P2X3 and/or P2X2/3-Rs is consistent with the original report by (Buller et al., 1996) that suramin prevented AVP neuron activation by moderate haemorrhage, because the P2X2, P2X3 and P2X2/3-Rs are also sensitive to suramin (Table 1).

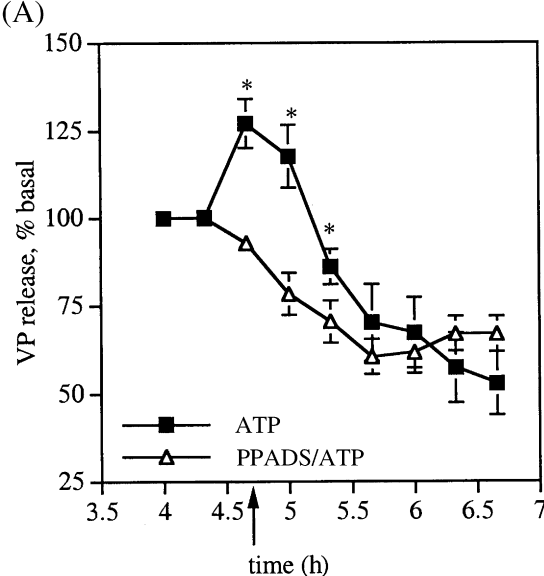
P2Y-R subtypes

The P2Y-Rs are G-protein-coupled receptors with seven transmembrane segments. There are eight mammalian P2Y-R subtypes that are coupled to either Gq or Gi (von Kugelgen, 2006). Electrophysiology studies provided evidence for P2Y2-Rs in SON neurons (Hiruma and Bourque, 1995), and Gi-coupled P2Y-R mediated Ca^{++} responses in pituicytes (Troadec et al., 1999). In our hands, Ca^{++}-imaging experiments provided evidence for prominent expression of P2Y1-R in SON as well as lower incidence of P2Y2 or 4-R, and low expression of P2Y6: (1) ATP continued to induce an increase in intracellular Ca^{++} ($[Ca^{++}]_i$) in the absence of extracellular Ca^{++} that was blocked by depletion of intracellular Ca^{++} stores with thapsigargin (TG) and (2) the P2Y1-R agonist, 2 methylthio-ADP (2-MeS-ADP) induced a large

Table 1. Characteristics of P2X-R subtypes in SON (North, 2002)

Subtype	In SON?		Blocked by 10 μM PPADS	Blocked by 30 μM suramin	Characteristics	
	mRNA[a]*	Pharmacol./other			Desensitize?	Pore?
P2X2	Yes	ATP-γ-S;[b] In NL[c]	Yes	Yes	Slow	Yes
P2X3	Yes*	2MeSATP[b] αβMeATP[d]	Yes	Yes	Fast	
P2X2/3	Yes/yes	αβMeATP	Yes	Yes	**No**	Yes
P2X4	Yes*	ATP-γ-S[b]	**No**	**No**	Slow	Yes
P2X6	Yes					
P2X7	Yes*	BBG[e]	**No**	**No**	**No**	Yes

[a]mRNA by RT-PCR in SON punches (Shibuya et al., 1999): *mRNA for P2X6 was detected (Shibuya et al., 1999); however, in expression systems, ATP induces only very small or undetectable currents via these subtypes. mRNA expression was most prominent for P2X3, 4 and 7.
[b]Ca^{++} imaging in isolated SON neurons (Shibuya et al., 1999).
[c]Ca^{++} imaging of isolated nerve terminals from neural lobe (NL; Troadec et al., 1998).
[d]Intracellular electrophysiological recordings from HNS explants (Hiruma and Bourque, 1995).
[e]Brilliant blue G (BBG), a selective P2X7-R antagonist at 30 μM, inhibited AMPA-R insertion in magnocellular neurons (Gordon et al., 2005).



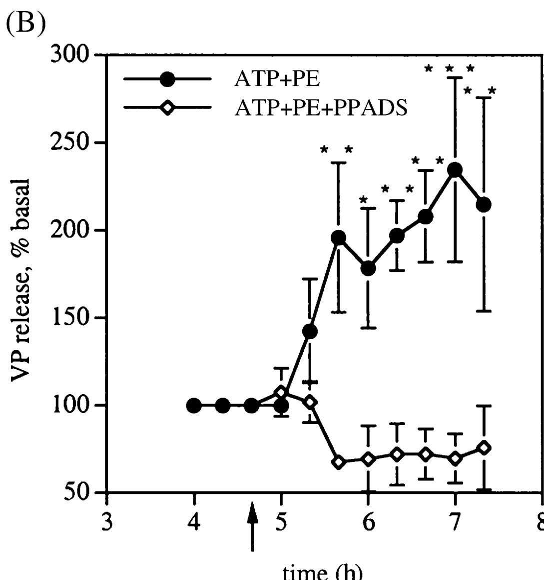


Fig. 5. Effect of 10 μM PPADS on AVP response to ATP (A) and ATP + PE. PPADS blocked the transient response induced by ATP alone (A) as well as the sustained response induced by ATP + PE (B). Adapted with permission from Kapoor and Sladek (2000).

increase in $[Ca^{++}]_i$ that accounted for 80% of the response to a cocktail of P2Y-R agonists (Fig. 6; Song et al., 2007). The role of P2Y-Rs in ATP stimulation of AVP/OTX release and the synergistic response to ATP + PE remains to be assessed.

Receptor signalling cascades activated by ATP+PE: role of $[Ca^{++}]_i$

Having demonstrated that P2X-Rs are required for the ATP + PE synergistic stimulation of AVP/OTX, we hypothesized that the ATP + PE synergism might reflect convergence of the intracellular signalling cascades triggered by ATP-initiated opening of P2X-gated ion channels and PE activation of the Gq/11 α1-Rs. As shown in Fig. 7, both pathways increase $[Ca^{++}]_i$, but via different pathways: one (P2X-R) by allowing influx of extracellular Ca^{++} and the other (α1-R) by initiating IP3-dependent release of Ca^{++} from intracellular stores. This convergence might augment elevated $[Ca^{++}]_i$ which, in turn, might recruit additional Ca^{++}-sensitive mechanisms. We were able to use AVP release from HNS explants as an end point to demonstrate that two components of the α1-R activated Gq/ll signalling cascade, release of Ca^{++} from intracellular stores and activation of protein kinase C (PKC), are required for the augmented and sustained increase in AVP release induced by ATP + PE (Kapoor and Sladek, 2000; Song et al., 2006). As shown in Fig. 8, pretreatment of HNS explants with TG to deplete intracellular Ca^{++} stores or the PKC inhibitor, bisindolylmaleimide, prevented ATP + PE-induced AVP release. Furthermore, inhibition of new gene expression by actinomycin also prevented the synergistic stimulation of AVP release by ATP + PE (Kapoor and Sladek, 2000). Thus, an augmented Ca^{++} signal may activate transcription factors resulting in up-regulation of specific genes. In order to directly evaluate the hypothesis that exposure to ATP + PE results in augmentation of the Ca^{++} signal, we directly monitored $[Ca^{++}]_i$ in SON neurons using HNS explants loaded with the Ca^{++}-sensitive dye, Fura-2AM. As shown in Fig. 9, Fura-2-loaded SON neurons are readily located and visualized from the ventral surface of HNS explants following incubation with Fura-2AM (see Song et al., 2006 for details). ATP, PE and ATP + PE, all caused the expected increase in $[Ca^{++}]_i$ (Fig. 9B), but the elevation in $[Ca^{++}]_i$ was extended in response to

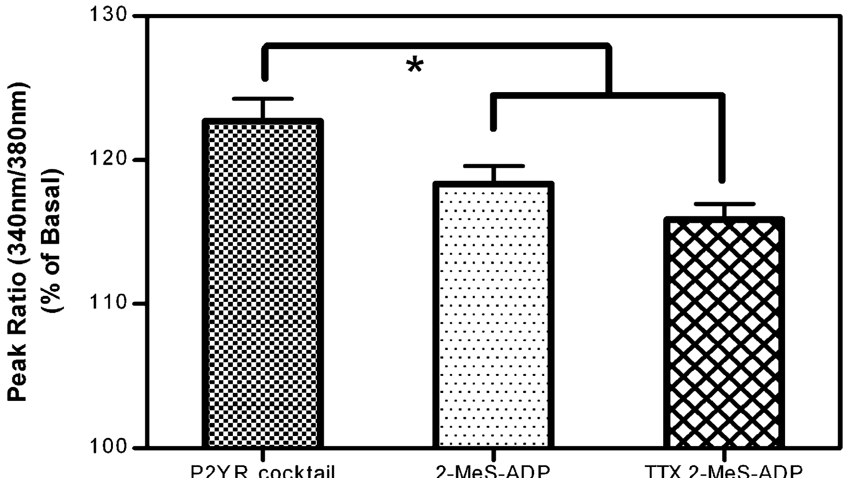


Fig. 6. A prominent Ca^{++} response is elicited in SON neurons by a cocktail of P2Y-R agonists (2-MethylS-ADP, 100 μM; UTP, 1 mM; and UDP, 1 mM) and by 2-MeS-ADP alone (a selective P2Y1-R agonist). 2-MeS-ADP is equally effective in the presence of TTX (to block action potential generation) and accounts for 80% of the response to P2Y-R activation. Adapted with permission from Song et al. (2007).

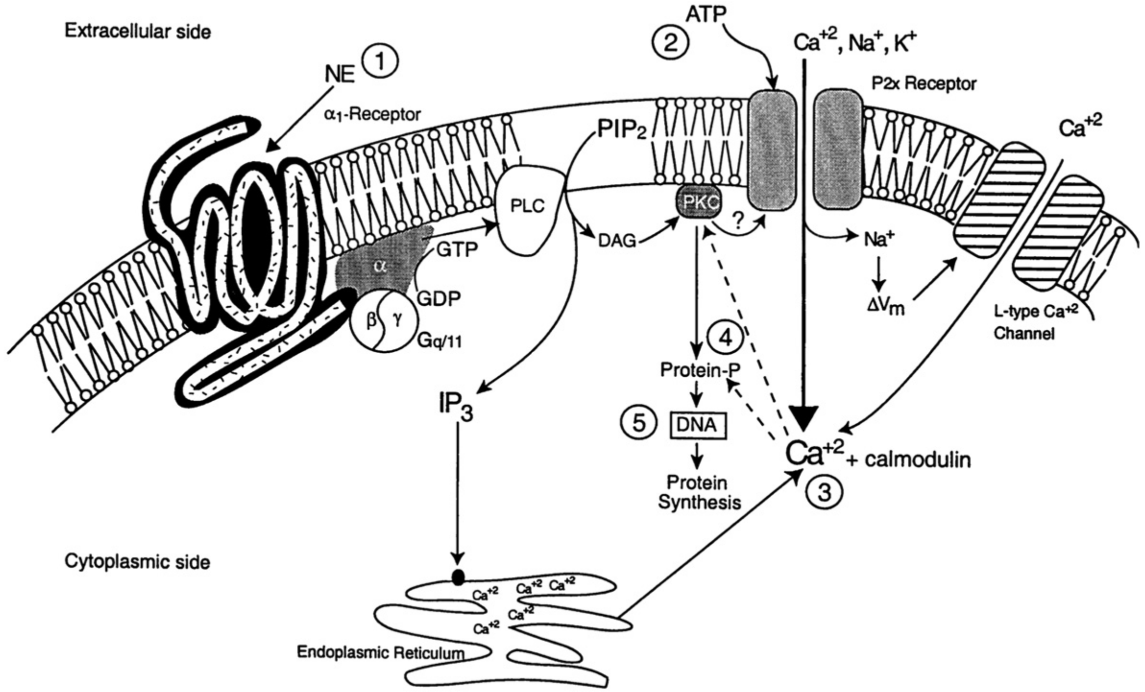


Fig. 7. Intracellular signal cascades initiated by activation of α1-receptor (1) and P2X-receptor (2). As a Gq/11-coupled receptor, activation of α1-Rs activates protein lipase C (PLC) leading to production of inositol triphosphate (IP3) which stimulates release of Ca^{++} from intracellular stores (3). Opening of the P2X-gated ion channel allows influx of extracellular Na^{+} and Ca^{++} (3). Depolarization initiated by Na^{+} influx opens voltage-sensitive Ca^{++} channels (L-type) resulting in further augmentation of $[Ca^{++}]_i$ (3). Convergence of these cascades on $[Ca^{++}]_i$ could recruit or enhance Ca^{++}-sensitive kinases resulting in phosphorylation of proteins (4) potentially resulting in altered receptor trafficking (desensitization) and/or gene expression (5). Adapted with permission from Kapoor and Sladek (2000).

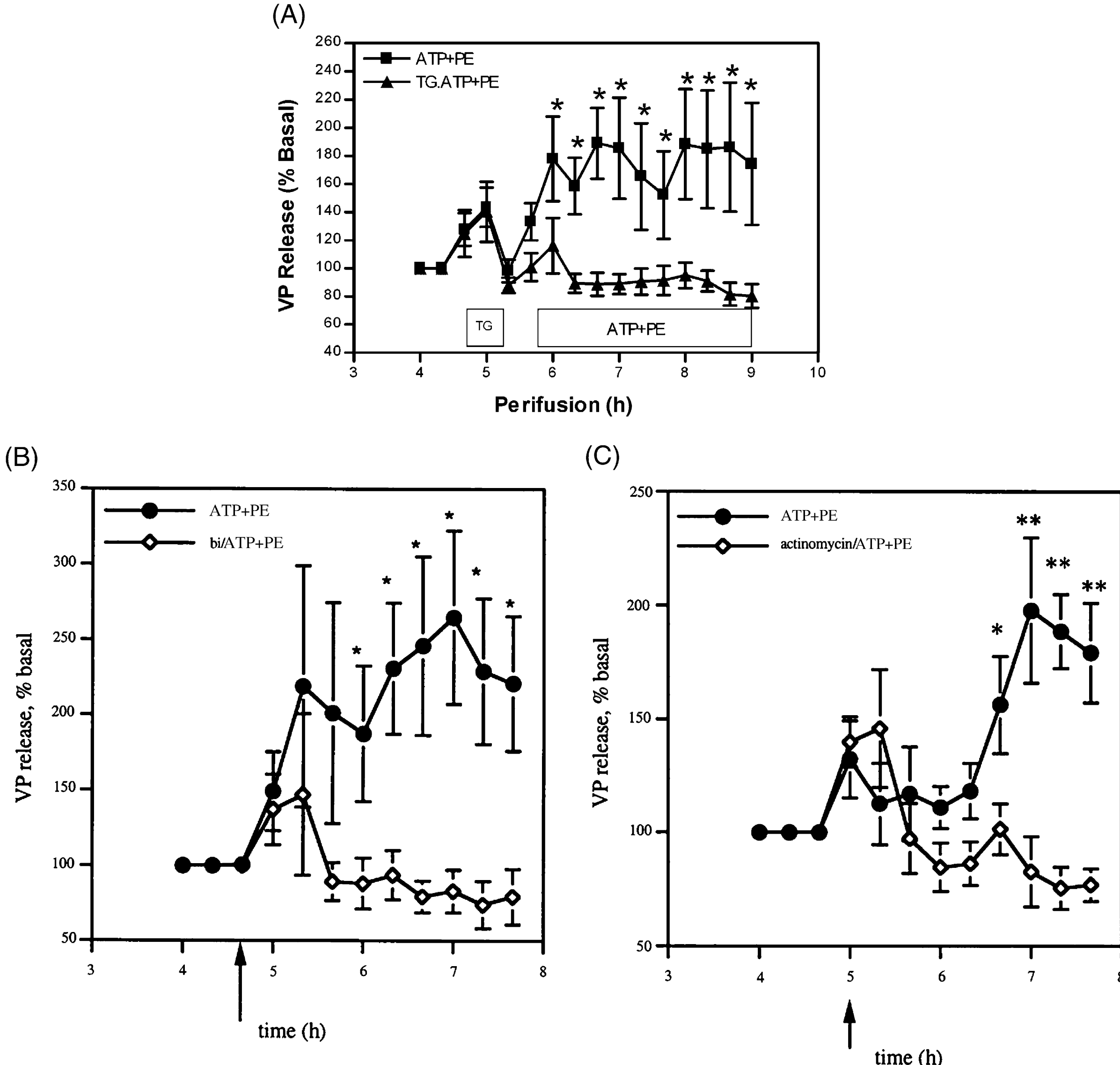
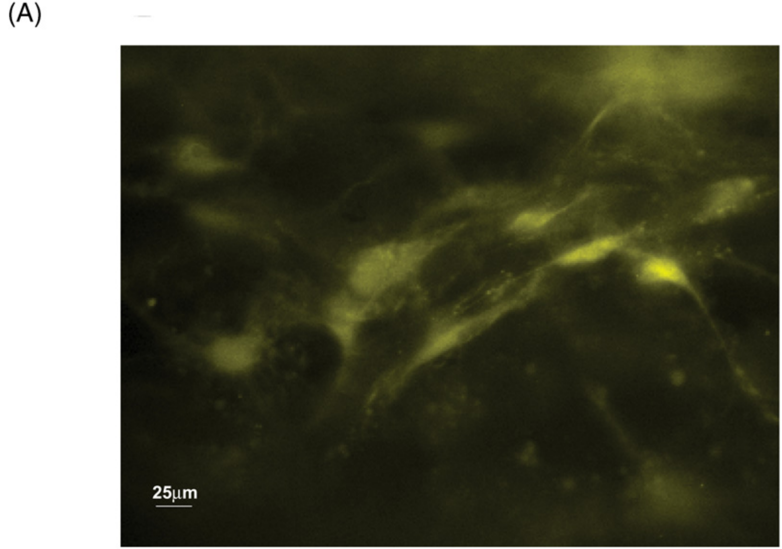


Fig. 8. The synergistic stimulation of AVP release by ATP + PE requires Ca^{++} release from internal stores (A), activation of PKC (B) and new gene expression (C). (A) Pretreatment with TG to deplete intracellular Ca^{++} stores prevented the prolonged and augmented stimulation of AVP release by ATP + PE. Note that DMSO was used as the vehicle for TG. Its presence in both groups during the 30-min exposure to TG increased the osmolality of the perifusate, resulting in the expected stimulation of AVP release. This effect was reversed, following the washout of DMSO and TG. Adapted with permission from Song et al. (2006). (B) The PKC inhibitory, bisindolylmaleimide (bi), prevented the synergistic stimulation of AVP release by ATP + PE. (C) Treatment with actinomycin to block gene transcription prevented the sustained component of ATP + PE stimulation of AVP release, but not the initial transient portion of the response. Adapted with permission from Kapoor and Sladek (2000).

ATP + PE (Song et al., 2006). Cells remained responsive to other stimuli, following washout of the ATP + PE (Song et al., 2006). Thus, the extended elevation in $[Ca^{++}]_i$ did not reflect cell dysfunction. Some of the imaged neurons were subsequently identified as AVP neurons based on mounting an increase in $[Ca^{++}]_i$ in response to AVP (Dayanithi et al., 1996; Song et al., 2006).

(A)



(B)

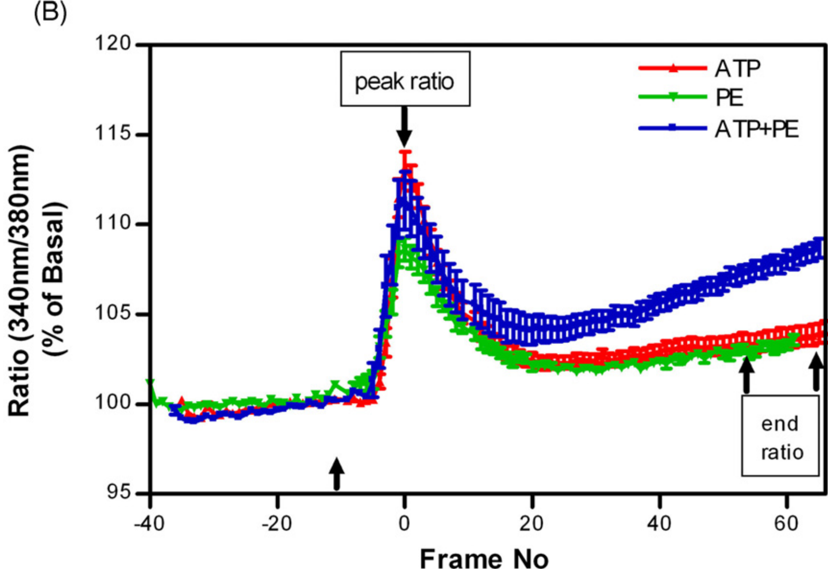


Fig. 9. (A) Fura-2-loaded SON neurons in an HNS explant preparation. (B) Live cell Ca^{++} imaging in Fura-2-loaded SON neurons. $[Ca^{++}]_i$ was increased by exposure to ATP, PE and ATP + PE (peak ratio). $[Ca^{++}]_i$ remained significantly elevated at the end of the approximately 4-min recording period (end ratio) following ATP + PE exposure compared to either agent alone ($p < 0.001$). Adapted with permission from Song et al. (2006). (See Color Plate 9.9 in color plate section.)

These observations confirmed our hypothesis that activation of multiple mechanisms for increasing $[Ca^{++}]_i$ results in a greater Ca^{++} signal for modulation of Ca^{++}-sensitive cellular mechanisms. This altered Ca^{++} signal might activate Ca^{++}-sensitive kinases resulting in phosphorylation of transcription factors to drive a change in gene expression, phosphorylation of receptors to alter desensitization characteristics or phosphorylation of other cellular proteins required for secretion.

Remaining issues and new hypotheses

What genes are regulated?

Since a shift from a transient response to ATP or PE alone to a sustained response is a hallmark of the ATP + PE synergism, and since P2X-R subtypes differ in their desensitization characteristics (Table 1), the P2X-R subtypes are interesting candidates for mediating the ATP + PE-induced shift from transient to sustained hormone release. Specifically, formation of P2X2/3 heterodimers could convert a transient response to ATP alone, mediated by either the slowly desensitizing P2X2-R or the rapidly desensitizing P2X3-R to one that does not desensitize. The sensitivity of both the ATP and ATP + PE responses to PPADS is consistent with this hypothesis, as well as the ability of suramin to block the activation of AVP neurons by moderate haemorrhage (Buller et al., 1996). Formation of heterodimers could be induced by increasing the number of either P2X2-R or P2X3-R subunits available. Thus, an increase in expression of one of these genes could alter the response to ATP. The requirement for PKC might reflect phosphorylation of P2X2-R subunits as this has been shown to modulate desensitization (Boue-Grabot et al., 2000).

P2X7-Rs are also interesting candidates for mediating a sustained response to ATP. They have been shown to alter synaptic function in SON (Gordon et al., 2005), are non-desensitizing and their activation by extended exposure to ATP results in formation of a pore large enough to flux molecules up to 900 Da (Table 1; North, 2002). Thus, pore formation can result in efflux or influx of small molecules such as nucleotides, glutamate and other neuroactive amino acids, serotonin and even small peptides. Since P2X7-Rs are not blocked by the concentration of PPADS that was effective in our experiments (10 μM), they cannot account for the full response to ATP in SON neurons. Involvement of P2X7-Rs in the sustained response to ATP + PE would require a switch in the P2X-R subtype responsible for initiating and sustaining the response. This possibility is supported by the observation that the sustained elevation in $[Ca^{++}]_i$ did not require continued presence of ATP + PE (Song et al., 2006). Thus, an initial augmentation of the $[Ca^{++}]_i$ signal can be postulated to be the signal that precipitates a change in gene transcription that subsequently leads to an augmented and sustained response to ATP. This possibility is supported by the delay in the augmented and sustained component of the response to ATP + PE (Fig. 1). However, a change in P2X-R gene expression is only one of many possibilities that might promote extended AVP/OTX responses. Subtypes of α1-R also differ in their rate of internalization (Chalothorn et al., 2002), and therefore a shift in the subtype of α1-R involved can similarly be postulated as a mechanism for the shift to a sustained response. Induction of the AVP and OTX genes might also participate, but due to the large hormone stores in the neural lobe, this is unlikely to be a prerequisite for sustained release in the time frame of our experiments. However, it may be important in pathological conditions associated with attenuation of AVP secretion resulting in shock (Oliver and Landry, 2007).

Are P2Y-Rs important for the synergistic response?

The finding that PPADS at a concentration that is selective for P2X receptors blocked ATP + PE-induced synergistic stimulation of AVP and OTX release from HNS explants demonstrates that activation of P2X receptors are required for the effect, but it does not eliminate the possibility that the P2Y receptors, which we subsequently demonstrated in SON, are also required (i.e. P2X-Rs are required, but may not be sufficient to induce synergism). Since P2Y-Rs are Gq/11-coupled receptors, their activation by ATP in SON neurons raises the question about their importance versus the α1-Rs, because both are coupled to Gq/11 and both induce release of Ca^{++} from internal stores. Perhaps the observed augmentation requires activation of multiple Gq/11-Rs or perhaps the receptors differ with respect to coupling to non-Gq-mediated signalling cascades [e.g. Gβγ, RhoA, MAPK/Erk, etc. (Hubbard and Hepler, 2006)]. P2Y-Rs have been associated with activation of MAPKs, PI3k, Akt and Ca^{++}-independent signalling cascades including P2Y1-R-mediated phosphorylation of ERK1/2

(May et al., 2006; Tran and Neary, 2006). Thus, evaluation of the role of P2Y-Rs in ATP + PE-induced synergism is important.

What is the physiological significance of the synergistic effect of ATP+PE?

Successful maintenance of cardiovascular homeostasis requires that the systems designed to maintain blood pressure have the capacity to respond to both acute decreases in blood return to the heart (e.g. orthostatic changes) and chronic conditions such as haemorrhage or dehydration. AVP is important in both situations due to its potent vasoconstrictor and antidiuretic actions. Thus, it is desirable to have mechanisms that allow for large and rapid increases in blood pressure in response to postural changes (moving from lying or sitting to standing) as well as sustained responses that can allow for fluid replacement. Very large, but transient increases in plasma AVP secretion occur throughout daily activity (Katz et al., 1979). In contrast, following haemorrhage, large and sustained elevations in plasma AVP are required to prevent cardiovascular collapse. The failure to maintain elevated plasma AVP is associated with the development of shock following haemorrhage and in other vasodilatory conditions (Oliver and Landry, 2007). The transition from transient AVP responses to ATP and PE alone to sustained AVP responses to ATP + PE described herein may underlie these important characteristics of AVP secretion in response to acute and chronic hypotension and hypovolemia. Identification of the receptors and cellular mechanisms underlying this transition will provide the information necessary to evaluate the importance of this phenomenon to cardiovascular homeostasis.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (R01 NS 27975 to CDS). The authors are grateful for the exceptional technical contributions of Ms. Wanida Stevens.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 10

Neuron–glia interactions in the rat supraoptic nucleus

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Abstract: The adult hypothalamo-neurohypophysial system undergoes a striking activity-dependent morphological remodelling that modifies the glial enwrapping of its magnocellular neurons. Although the functional consequences of such remodelling remain hypothetical, recent evidence has provided new insights into the repercussions of glial environment modifications on the physiology of magnocellular neurosecretory cells at the synaptic level. These studies have revealed that the reduced astrocytic coverage of magnocellular neurons occurring in the SON affects various functions in which astrocytes play key roles. These functions include uptake of neurotransmitters such as glutamate, restricting diffusion of neuroactive substances within the extracellular space and release of informative molecules known as gliotransmitters that act on neighbouring neurons to modulate synaptic transmission and excitability. Overall, our observations indicate that the neuron–glial anatomical reorganization leads to modifications of glutamatergic transmission that might be important for the physiology of the hypothalamo-neurohypophysial system.

Keywords: glutamate; spillover; diffusion; lactation; D-serine; LTP; astrocyte; oxytocin

Introduction

The hypothalamo-neurohypophysial system is a neuroendocrine system essential for animal survival. It is composed of magnocellular neurons located in the paraventricular (PVN) and supraoptic (SON) nuclei. These neurons synthesize the hormones oxytocin (OXT) and vasopressin (AVP) (Swaab et al., 1975). Whereas OXT plays a key role in reproductive functions like parturition and lactation, AVP is essential to body fluid and cardiovascular homoeostasis (Poulain and Wakerley, 1982). Magnocellular neurons send their axons to the neurohypophysis where OXT and AVP are secreted directly in the blood stream. In addition, these peptides can be released centrally in various brain regions including within the PVN and SON (Ludwig and Pittman, 2003) where they exert local regulatory actions (Kombian et al., 2002; Ludwig et al., 2002; Oliet et al., 2007). OXT and AVP secretion depends on the electrical activity of magnocellular neurons which is itself under the

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DOI: 10.1016/S0079-6123(08)00410-X

influence of excitatory glutamatergic and inhibitory GABAergic synaptic afferent inputs (Leng et al., 1999; El Majdoubi et al., 2000).

Under basal conditions of secretion, like in virgin rats, OXT neurons often occur in tightly packed clusters. In spite of their tight apposition, they are separated by neuropil elements including fine lamella-like processes of astrocytic origin (Theodosis, 2002). Interestingly, under conditions of strong neurohypophysial hormone secretion, such as during chronic dehydration, parturition and lactation, the hypothalamo-neurohypophysial system, and in particular its oxytocinergic portion, undergoes a remarkable anatomical remodelling that is reversible upon cessation of the stimulation (Myiata and Hatton, 2002; Theodosis, 2002). This remodelling is primarily characterized by a pronounced reduction of the astrocytic coverage of neurons and synapses. Thus, under certain physiological conditions, a diminished astrocytic coverage of neurons and synapses may impact the magnocellular system in view of the different functions ensured by glial cells in the central nervous system.

Work performed during the last decade has revealed that glial cells were an active component of the chemical synapse, leading to the emerging concept of the tripartite synapse (Volterra and Meldolesi, 2005; Haydon and Carmignoto, 2006). Among the different roles played by astrocytes, it is well admitted that they represent physical barrier to diffusion in the extracellular space (Syková, 2001) and that they are responsible for the clearance of neurotransmitters like glutamate and GABA (Schousboe, 2003). Furthermore, glial cells can detect synaptic activity through the expression of specific receptors at their surface whose stimulation can lead to the release of signalling molecules named gliotransmitters by analogy to neurotransmitters. In view of these functions, the change in glial environment might impact the concentration and range of action of both neuro- and glio-transmitters in the SON.

Three types of astrocytes are present in the SON. There are typical stellate astrocytes found interspersed in the nucleus (Montagnese et al., 1988; Bonfanti et al., 1993). The most numerous are radial-like astrocytes whose cell bodies are located in the ventral part of the nucleus. These cells send long processes oriented ventro-dorsally through the nucleus (Bonfanti et al., 1993). The last type of astrocytes, found in the ventral glia lamina (VGL) close to the subarachnoid space, is characterized by small and round cell bodies with few processes (Salm, 2000; Israel et al., 2003). Recent electrophysiological analyses performed in lactating and dehydrated animals have investigated the consequences of astroglial remodelling onto synaptic transmission in the SON and PVN (Oliet et al., 2001; Boudaba et al., 2003; Piet et al., 2004; Gordon et al., 2005; Panatier et al., 2006). Here, we will review the results obtained in lactating and dehydrated rats and their putative repercussions for the physiology of the hypothalamo-neurohypophysial system.

Diffusion properties in the SON

Clearance of neurotransmitters depends on degradation, uptake and diffusion. Indeed, fine astrocytic processes hinder diffusion of molecules in the extracellular space (Syková, 2001). Glial wrapping of synapses and neuronal elements is therefore likely to limit the amount of transmitter escaping from the synaptic cleft, a phenomenon known as “spillover”. As a consequence, the astrocytic environment of neurons appears to govern cross-talk between adjacent synapses and intercellular communication relying on extrasynaptic (or volume) transmission (Isaacson, 2000; Kullmann, 2000). The degree of astrocytic coverage of neurons, therefore, is likely to influence the diffusion properties in the extracellular space. This was elegantly demonstrated in the SON where diffusion parameters were measured in virgin and lactating rats (Piet et al., 2004). To this end, an extracellular marker, tetramethylammonium (TMA^+), was injected in the tissue and its concentration was detected at short distance by a TMA^+ selective electrode. With this approach, it is possible to determine tortuosity, which is an index of diffusion hindering in the tissue, and volume fraction, which reflects the proportion of tissue volume available for diffusion. In virgin rats, diffusion was found to be anisotropic since

tortuosity was hindered less along the ventro-dorsal plane, a result probably reflecting the ventro-dorsal orientation of most astrocytic processes in this nucleus (Salm, 2000; Israel et al., 2003). In the SON of lactating rats, both tortuosity and volume fraction were significantly reduced and diffusion became isotropic (Piet et al., 2004). This is likely to be a consequence of the reduction of astrocytic coverage of neurons and the associated increased in the proportion of directly juxtaposed neuronal surfaces. These results demonstrate that the neuronal–glial remodelling occurring in the SON of lactating rats not only modifies geometry but also facilitates diffusion in the extracellular space. This may modify diffusion of molecules, including neurotransmitters as well as OXT and AVP, which are also released in the SON (Ludwig and Pittman, 2003). More generally, glial withdrawal associated with the anatomical remodelling of the hypothalamo-neurohypophysial system would increase the concentration and the range of action of any neuroactive substances released in the tissue (neurosteroids, peptides, endocannabinoids…), except, of course, those released from the glial processes that have retracted.

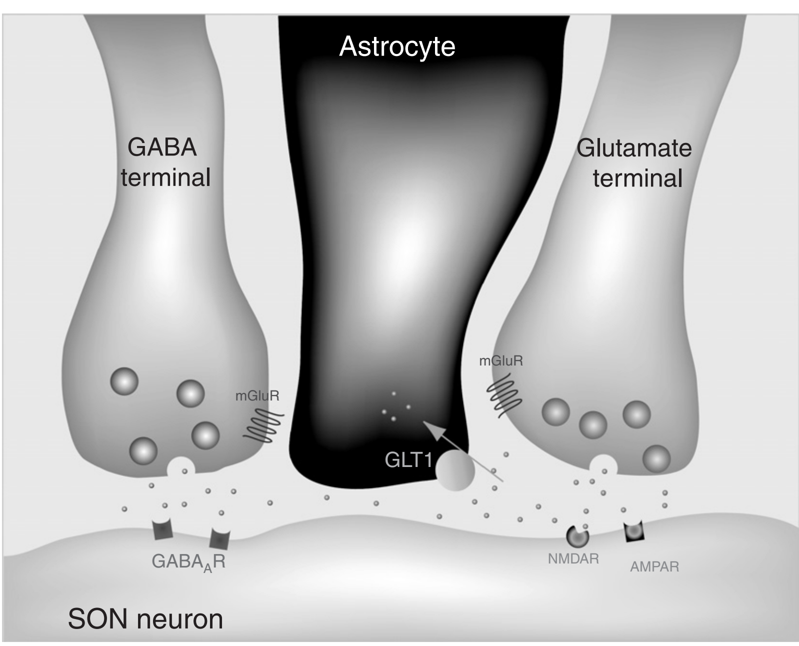
Glial contribution to glutamatergic and GABAergic transmission in the SON

Besides the key role played by astrocytes in hindering the diffusion of molecules in the extracellular space, astrocytes play a prominent role in the clearance of synaptically-released glutamate (Danbolt, 2000). This is ensured thanks to the high-affinity glutamate transporters GLT-1 and GLAST located on their plasma membrane. There is compelling evidence indicating that astrocytic GLT-1 subtype of transporter is responsible for most, if not all, of the uptake of synaptically-released glutamate in the central nervous system (Bergles et al., 1999; Danbolt, 2000). Whereas local variations of glutamate concentrations at the vicinity of release sites can affect synaptic transmission at both pre-and postsynaptic levels (Scanziani et al., 1997; Min et al., 1999; Mitchell and Silver, 2000; Isaacson, 2000), a persistent deficiency in glutamate uptake may lead to excitotoxicity through the chronic activation of glutamate receptors.

The contribution of astrocytes to excitatory transmission in the SON can be assessed through the use of specific glutamate transporter antagonists. Under conditions where the glial coverage of SON neurons is intact (virgin or normally-hydrated rats), inhibition of GLT-1 transporters caused a depression of both glutamate (Oliet et al., 2001; Boudaba et al., 2003) and GABA release (Piet et al., 2003). Detailed electrophysiological analyses revealed that this inhibitory action of transporter blockers resulted from glutamate build up in the extracellular space and the resulting activation of presynaptic group III metabotropic glutamate receptors (mGluRs). Since these mGluRs are negatively coupled to transmitter release, their activation induces a reduction of the probability of synaptic release (Oliet et al., 2001).

Experiments were subsequently carried out in lactating (Oliet et al., 2001) and chronically dehydrated (Boudaba et al., 2003) rats to assess consequences of the anatomical remodelling on mGluR-mediated modulation of glutamatergic transmission. They revealed that glutamate clearance was delayed under conditions of reduced glial coverage of magnocellular neurons. As a consequence, glutamate accumulates in the extracellular space enhancing the tonic activation of presynaptic mGluR and thereby resulting in an augmented negative feedback exerted by glutamate on its own release (Oliet et al., 2001) (Fig. 1). Thus, astrocytes control the level of the negative feedback exerted by ambient glutamate on its own release at excitatory synapse. This mechanism is likely to affect glutamatergic inputs differentially according to their firing rate. Moderately active inputs will be inhibited whereas the activity-dependent facilitation of transmitter release occurring during high-frequency trains of action potential may, to some extent, overcome the mGluR-mediated presynaptic inhibition.

Glutamate can diffuse and then activate receptors near its releasing site as GABAergic terminals. At those inhibitory terminals, ambient glutamate does not exert a tonic modulation in the SON of virgin rats (Piet et al., 2003). This is not surprising since the concentration of glutamate drops as



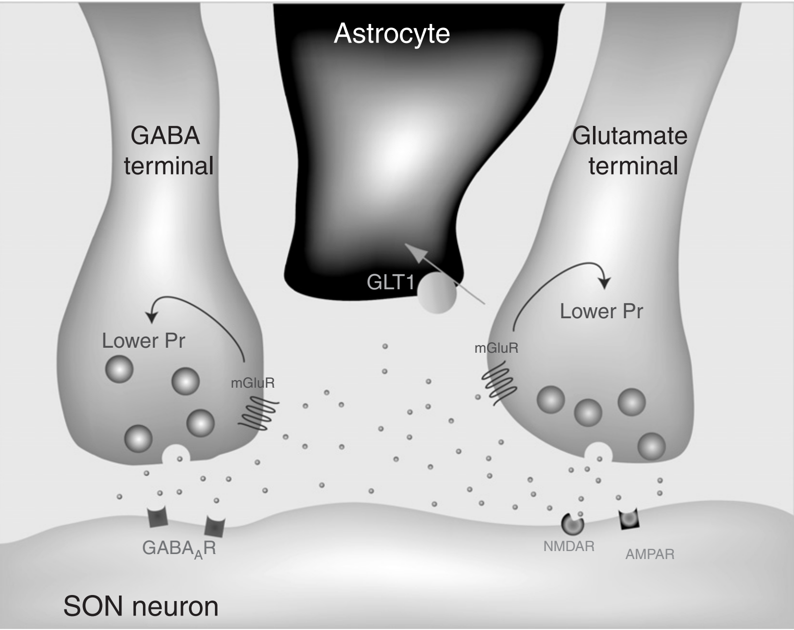


Fig. 1. Astrocytic processes control glutamate concentration and diffusion in the extracellular space. Under control conditions (upper panel) glial processes ensure glutamate clearance thanks to GLT1 transporters expressed at their surface. As a consequence, the tonic inhibition mediated by presynaptic mGluRs on these excitatory inputs is restrained. Furthermore astrocytes represent a physical barrier hindering the diffusion of the excitatory amino acid, thereby limiting the access to adjacent GABAergic synapses and the mGluRs localized presynaptically. Conversely, under stimulated conditions (lower panel), glial withdrawal enhanced the tonic activation of mGluRs on glutamatergic inputs and facilitated heterosynaptic depression of GABA release mediated through glutamate spillover.

distance from release sites increases, and is likely to be ineffective for the activation of remote mGluRs. It is possible, however, that the elevation of extracellular glutamate concentrations associated with the anatomical remodelling occurring during lactation or chronic dehydration might be sufficient to permit such an intersynaptic modulation. This does not seem to be the case (Piet et al., 2003). Taken together, these data indicate that the elevation of extracellular glutamate concentrations resulting from the reduced astrocytic coverage of supraoptic neurons is a local phenomenon circumscribed to glutamatergic inputs.

That ambient glutamate does not affect mGluRs on inhibitory terminals in the SON of either virgin or lactating animals is consistent with previous studies that investigated the modulation of GABA-A receptor-mediated transmission by glutamate spillover in different brain areas (Mitchell and Silver, 2000; Semyanov and Kullmann, 2000; Satake et al., 2000). Such types of heterosynaptic modulation are sensitive to the activity of glutamatergic inputs since it is observed only in response to strong and/or sustained glutamate release. Indeed, the extracellular concentration of glutamate has to reach sufficient levels to saturate the transporter system and diffuse away to affect adjacent synapses. A similar form of crosstalk between glutamatergic and GABAergic synapses is observed in the SON under conditions where glutamate release is induced by brief high-frequency trains of stimuli (Piet et al., 2004). This results in the depression of GABA-A receptor-mediated synaptic currents caused by glutamate spillover activating group III mGluRs on inhibitory terminals (Fig. 1). Because this type of modulation relies on the diffusion of glutamate in the extracellular space, it is likely to be affected by glial withdrawal. In agreement with this hypothesis, mGluR-mediated inhibition of GABA release by glutamate spillover is largely enhanced in the SON of lactating rats (Piet et al., 2004) (Fig. 1).

Such enhanced heterosynaptic inhibition of GABA transmission could have important consequences on transmission and integration of excitatory signals by SON neurons. By reducing GABA release, glutamate spillover would diminish the weight of inhibition, thereby disinhibiting locally OXT cells and thus favouring excitation.

Gliotransmission in the SON and modulation of NMDA receptors

It is now acknowledged that glial cells can modulate synaptic transmission by releasing gliotransmitters (Volterra and Meldolesi, 2005; Haydon and Carmignoto, 2006). One of them is D-serine, a D-amino acid acting as a ligand at the glycine-binding site of NMDA receptors (NMDARs). This is of strong relevance for the central nervous system since NMDA receptors play an important role in fast excitatory transmission and long-term synaptic plasticity. NMDAR activation depends on the binding of glutamate and of a co-agonist like glycine or D-serine. Interestingly, high levels of D-serine are present in SON astrocytes. Consistent with reports from the hippocampus and retina (Mothet et al., 2000; Yang et al., 2003; Stevens et al., 2003), NMDAR-mediated synaptic responses in magnocellular neurons were dramatically reduced in hypothalamic slices treated with D-amino acid oxidase, (DAAO), an enzyme specifically degrading D-serine. The inability of glycine oxidase (GO), which specifically degrades glycine, to impact NMDAR currents provides further support for the assertion that D-serine, and not glycine is the endogenous co-agonist of NMDARs in this system (Panatier et al., 2006). If glial cells contribute to NMDAR activity, it is very likely that the responses mediated by these receptors will be affected under conditions of reduced astrocytic coverage. In agreement with this hypothesis, NMDAR-mediated synaptic responses are decreased in the SON of lactating animals. That these responses can be recovered when the media is supplemented with saturating concentrations of D-serine provides the final demonstration that glial-derived D-serine is the endogenous ligand for NMDARs in the SON.

Under these conditions when D-serine concentration within the synaptic cleft is reduced, the number of NMDARs available for synaptic activation is also reduced, resulting in dramatic changes in the induction of activity-dependent

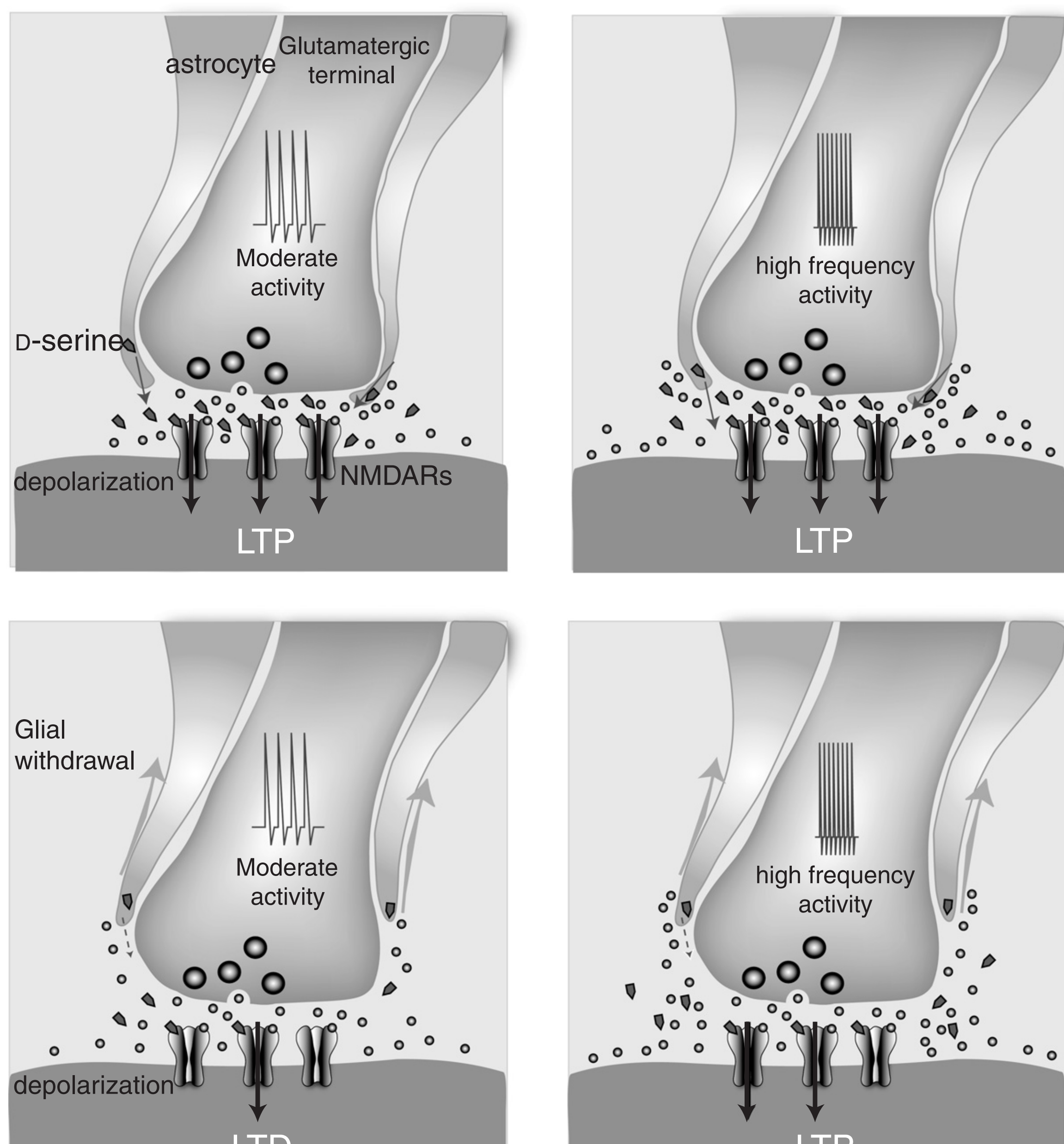


Fig. 2. The glial environment of neurons governs the direction and magnitude of long-term synaptic plasticity. Under control conditions (upper panels), moderate activity of glutamatergic inputs combined with postsynaptic membrane depolarization (left) induces LTP in SON neurons. A similar synaptic strengthening is observed in response to high-frequency trains of stimuli (right). Under stimulated conditions (lower panel), glial withdrawal leads to a deficit in D-serine within the synaptic cleft. Consequently, moderate gluatamatergic input activity paired with membrane depolarization no longer induces LTP but triggers LTD (left). This is due to the limited amount of NMDARs available for activation under this situation. Interestingly, if a stronger protocol of stimulation is used (right), then LTP can be restored probably because the number of NMDARs activated is now sufficient to trigger the LTP pathway.

plasticity like long-term potentiation (LTP) and long-term depression (LTD) (Panatier et al., 2006). Conditions of low astrocyte coverage are associated with a shift in the activity-dependence of long-term synaptic changes towards higher activity values. Simply put, experimental protocols that in control conditions caused LTP now elicit cause LTD. This is consistent with reports from both the CA1 (Cummings et al., 1996) and CA3 (Bains et al., 1999) regions of the hippocampus demonstrating a switch to LTD when high frequency stimulation is applied in the presence of partial NMDARs blockade. The simplest explanation for this switch in the direction of the plasticity is that a reduction in the number of NMDARs solicited during the induction protocol translates into a smaller postsynaptic Ca^{2+} rise. This is no longer sufficient to trigger LTP, but is appropriate for the manifestation of LTD (Fig. 2). In hypothalamic slices from lactating rats, application of saturating concentrations of D-serine increased the number of NMDARs available for activation this situation and entirely reversed this effect (Panatier et al., 2006). Such astrocytic-dependent metaplasticity is likely to prevail at all synapses where endogenous D-serine is the co-agonist of NMDARs and could be of prime importance under physiological and/or pathological conditions where the anatomical interaction between neurons and glia is modified.

Conclusions

Taken together, the data reviewed here, provide new insights on the functional consequences of the anatomical remodelling occurring in the SON during lactation or chronic dehydration. In particular, modification of the astrocytic environment of neurons appears to modify both synaptic and extrasynaptic transmission mediated by glutamate. This is directly related to the presence on glial cells of transporters that clear away the excitatory amino acid and to the fact that fine astrocytic processes dictate diffusion properties in the extracellular space. Beside the modulation of presynaptic mGluRs, variations in the concentration and diffusion of glutamate in the SON may also affect other glutamate receptors whose activity can strongly influence neuronal electrical behaviour. Similarly, the action of other transmitters is likely to be influenced by the astrocytic coverage of neurons. Another main consequence of this anatomical reorganization is the reduced concentration of D-serine within the synaptic cleft. This has direct consequence on NMDA receptor activity and NMDA-dependent processes. By analogy, it is very likely that the modulatory actions on SON neurons of substances released by astrocytes like taurine (Hussy, 2002) or ATP (Gordon et al., 2005) are also limited by the increased distance separating glial processes from neuronal elements. Indeed, this has been already demonstrated for ATP in dehydrated animals (Gordon et al., 2005). Through the identification of the consequences of the reduced astrocytic coverage onto synaptic transmission and plasticity, it is now possible to formulate some hypotheses regarding the physiological role of the anatomical remodelling occurring in the hypothalamo-neurohypophysial system. The general scheme coming out from the results reviewed here is that the remodelling serves to isolate OXT neurons from non-pertinent stimuli at a period where the only relevant stimulus is pup suckling. In this model, the amount of OXT available for secretion in the blood stream will be preserved for milk ejection making pup survival the priority for the OXT system. This working hypothesis, however, still needs to be verified experimentally.

Abbreviations

AVP	Vasopressin
DAAO	D-amino acid oxidase
GABA	gamma-aminobutyric acid
GO	glycine oxidase
LTD	long-term depression
LTP	long-term potentiation
mGluR	metabotropic glutamate receptor
NMDA	*n*-methyl-D-aspartic acid
OXT	oxytocin
PVN	paraventricular nucleus
SON	supraoptic nucleus
TMA^+	tetramethylammonium
VGL	ventral glia lamina

Acknowledgements

Work in the authors' laboratory is supported in part by grants from Inserm, Human Frontier Science Program, ANR, FRC and the Conseil Régional d'Aquitaine. SHRO is the recipient of a NARSAD independent investigator award. Aude Panatier and Richard Piet were funded by studentships from the French Ministry of Education and Research.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 11

Dynamic synapses in the hypothalamic-neurohypophyseal system

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Abstract: The release of vasopressin and oxytocin from the posterior pituitary is tightly coupled to the activity of magnocellular neurosecretory cell (MNC) bodies in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. These cell groups exhibit distinct patterns of activity which are regulated by a combination of synaptic inputs and intrinsic properties. The postsynaptic currents (intrinsic properties) that shape these bursts have been described extensively but examinations of the contributions of synaptic input to activity patterns in these cells are relatively sparse. Although the synaptic release of glutamate is necessary to initiate and sustain bursting, precisely how a brief depolarization associated with a synaptic current would ignite such a prolonged postsynaptic discharge is not clear. Here, we review recent work from our laboratory showing that unlike the majority of synapses in the brain, glutamate synapses onto MNCs release transmitter in an asynchronous fashion following a presynaptic action potential. This input is integrated by the postsynaptic neuron and may serve to activate the postsynaptic conductances necessary for the induction of patterned activity.

Keywords: glutamate; presynaptic; asynchronous; plasticity; PVN; magnocellular; burst

In the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, vasopressin (AVP) neurons are intermingled with neurons that produce oxytocin (OT). The axons of both cell groups project to the posterior pituitary, where individual fibres branch into roughly 2000–10,000 nerve endings (Leng et al., 1999). Each of these boutons contains dense core vesicles filled either with AVP or OT. Unlike traditional synaptic terminals which often require only a single action potential in the presynaptic terminal to cause the exocytosis of a vesicle(s), MNC axon terminals require long bursts of action potentials to effectively release hormone into the blood (Leng et al., 1999). In vivo, OT and AVP cells show different spontaneous firing patterns that permit efficient hormone secretion. OT cells, in response to stimulation (suckling or during parturition), fire in short, high-frequency bursts (2–4 s, 40–80 Hz) (Wakerley et al., 1973). AVP cells, on the other hand, fire at very low frequencies and show intermittent phasic bursts in which activity (firing in a range of 7–15 Hz) and quiescence alternate with a period of approximately 1–2 min. In rats, hyperosmolality elicits an increase in phasic firing in AVP neurons specifically (Poulain et al., 1977; Wakerley et al., 1978). Although OT neurons

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DOI: 10.1016/S0079-6123(08)00411-1

also increase their firing rate during a hypertonic challenge, they do not exhibit phasic firing (Brimble and Dyball, 1977; Poulain et al., 1977). Previous studies in vitro have shown that the release of both OT and AVP increases with neuronal firing rate (Dreifuss, 1973), but is optimal during bursting activity (Dutton et al., 1978; Bicknell and Leng, 1981). How these prolonged bursts are initiated, however, is a matter of some debate. In vivo there are clear demonstrations that synaptic glutamatergic input is essential for these phasic bursts (Nissen et al., 1995; Brown et al., 2004), yet there have been few investigations that have attempted to explicitly understand how synaptic glutamatergic input contributes to phasic activity. In addition to potential synaptic contributions, there is a critical role for the summation of depolarizing after potentials (DAPs) following a brief burst of action potentials in the maintenance of phasic activity (Roper et al., 2003, 2004; Brown and Bourque, 2006). The mechanism responsible for the generation of these DAPs is contentious (Bourque et al., 1998), but recent reports indicate that they may result from the activation of Ca^{2+}-sensitive non-specific cation conductances in magnocellular neurosecretory cells (MNCs) (Ghamari-Langroudi and Bourque, 2002). While DAPs are important, it is not known whether synaptic activity in particular can activate DAPs and shape this patterned discharge. To begin to address this question requires, first, a capitulation of the basic rules of synaptic transmission at synapses that relay information to MNCs.

Synaptic physiology of glutamate synapses on MNCs

The synapse is a specialized structure that allows for the chemical communication between cells in the nervous system. Although all synapses serve this same basic function, they do so in a number of different ways. For example, in auditory pathways, synapses are geared to favour precision. They transmit high-frequency information with great fidelity. By contrast, in the neuromuscular junction, synapses are designed to generate large, postjunctional potentials, while in some regions of the cortex, excitatory synapses are only effective if a number of them are activated simultaneously. This diversity, to a large degree, reflects the function of the system in which these synapses exist. In MNCs, precise spike timing in response to an afferent input is not critical for hormone release. Instead, these cells must integrate synaptic input from multiple afferent nuclei to generate patterns of activity that can support the release of a bolus of hormone into the blood. This integration is aided, in part by the high input resistance and slow membrane time constant of these cells (Tasker and Dudek, 1991). Glutamate is the primary fast excitatory neurotransmitter in the PVN (van den Pol et al., 1990), eliciting its effects through the activation of postsynaptic AMPA (Wuarin and Dudek, 1993) and NMDA (Bains and Ferguson, 1997, 1999) receptors. Since MNCs serve as the end effectors for a neural feedback circuit that orchestrates the central nervous system (CNS) response to perturbations in physiological homeostasis, the synapses onto these cells constitute the final integration of signals before the release of OT and AVP. Consequently, mechanisms that modulate either the rate at which glutamate-filled vesicles are released, or the postsynaptic efficacy of an individual quantum of neurotransmitter will impact neuroendocrine output. The efficacy of this transmission can be altered by various neuromodulators through transient changes in either presynaptic neurotransmitter release probability (Bains and Ferguson, 1997; Kombian et al., 1997, 2000a, b, 2001; Schrader and Tasker, 1997; Daftary et al., 1998; Harayama et al., 1998; Inenaga et al., 1998; Oliet and Poulain, 1999; Shibuya et al., 2000; Boudaba et al., 2003; Gordon and Bains, 2003; Baimoukhametova et al., 2004; Hirasawa et al., 2004; Di et al., 2005) and/or postsynaptic changes in AMPA receptor function and the gating of voltage-gated ion channels (Randle et al., 1986; Hiruma and Bourque, 1995; Hirasawa et al., 2003; Brown et al., 2004).

The majority of these studies, however, operate on the assumption that a presynaptic action potential triggers the probabilistic and synchronized release of vesicles filled with glutamate within a few milliseconds of the presynaptic depolarization. While this is clearly the case for the majority of synapses in the brain (Lisman et al., 2007), it is not the case for glutamate synapses onto MNCs in

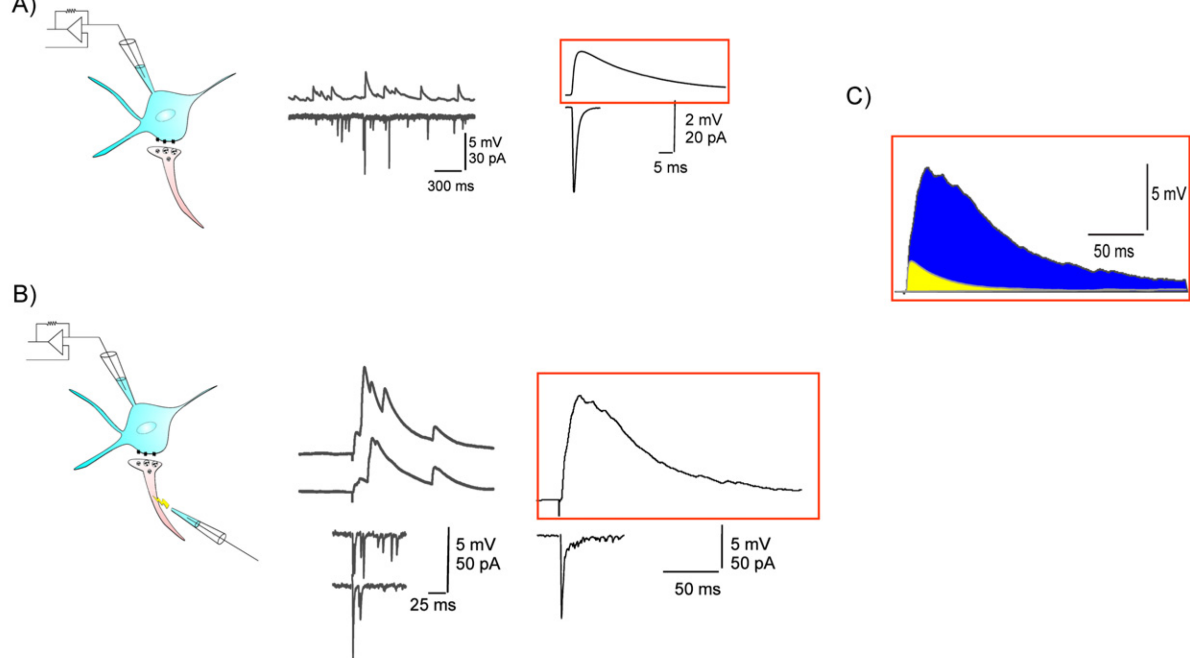

Fig. 1. Asynchronous release elicits a large postsynaptic depolarization in MNCs. (A) Spontaneous EPSCs in MNCs have a fast rise and decay time. These quantal release events can produce large EPSPs that have a long decay time owing in part to the long membrane time constant of MNCs. (B) A single presynaptic action potential evokes the asynchronous release of multiple glutamate vesicles. In current clamp, these single events summate efficiently to produce a large and prolonged EPSP. (C) An evoked asynchronous EPSP overlaid with a single quantal EPSP. Portions of figure adapted with permission from Iremonger and Bains (2007).

PVN (Fig. 1). In fact, we have shown recently that the majority (>60%) of glutamate synapses onto MNCs exhibit a form of delayed or asynchronous release in response to a single presynaptic action potential (Iremonger and Bains, 2007). Here, a single action potential triggers either zero, one or multiple release events during a temporal window that may extend for up to 100 ms following the presynaptic action potential (Fig. 2). Synaptically evoked waveforms kinetically similar to this delayed release have been reported previously in the PVN but, at that time, were ascribed to be indicative of recruitment of polysynaptic glutamatergic circuits (Boudaba et al., 1997). Our experiments, however, show clearly that single synaptic stimuli can elicit all-or-none delayed responses, consistent with the idea that this delayed release reflects the activation of terminals that can release in an asynchronous fashion and does not reflect the activation of local glutamate circuits (Iremonger and Bains, 2007). Importantly, it appears that single neurons can receive inputs that are either exclusively synchronous, exclusively asynchronous or both synchronous and asynchronous. These observations of diverse presynaptic release profiles are consistent with the idea that the mode of release is regulated by the presynaptic neuron and is not dependent on the target neuron. This is an important consideration since release probability at inhibitory GABA synapses onto MNCs is regulated by the target cell itself (Oliet et al., 2007). Interestingly, while we consistently observe asynchronous release onto MNCs, we have rarely observed asynchronous release onto neighbouring parvocellular neurosecretory cells (<10% of synapses on over 300 cells tested). Consistent with our hypothesis that asynchronous release is an integral component of patterned activity, there are no reports of episodic discharges in parvocellular neurosecretory cells.

In the vertebrate CNS, similar asynchronous release profiles in response to a single action potential have been described previously at the granule

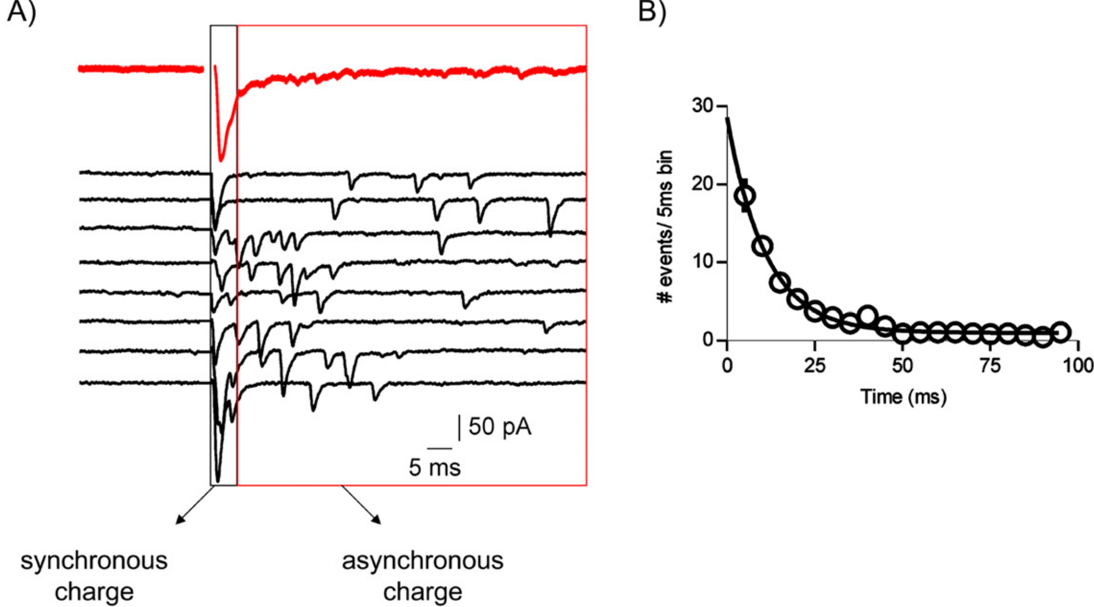

Fig. 2. Single presynaptic action potentials evoke both synchronous and asynchronous release. (A) Top trace (red) shows the average EPSC taken from 30 trials. Bottom traces (black) show individual trials. In some trials, a fast, synchronized EPSC is evoked immediately after the presynaptic stimuli. In other trials, the fast, synchronized EPSC is virtually absent, and only delayed, asynchronous release is present. Stimulation artefacts have been removed for clarity. (B) Graph showing the number of delayed release events from 5 to 100 ms after the presynaptic stimuli (5 ms bins, total number of events from 30 trials). The number of delayed release events decays exponentially with a time constant of 10.9 ms ($n = 23$). Figure is adapted with permission from data in Iremonger and Bains (2007).

cell to stellate cell synapse in the cerebellum (Atluri and Regehr, 1998), in the calyx of Held (Wu et al., 1999) and at inhibitory synapses from interneurons to pyramidal cells in the hippocampus (Hefft and Jonas, 2005). Here, the release of neurotransmitter-filled vesicles lasts for tens to hundreds of milliseconds after a presynaptic action potential. Even synapses that do not show overt asynchronous release in response to a single action potential can often display robust episodes of prolonged release when the presynaptic nerve terminal is activated in a repetitive fashion (Otsu et al., 2004; Otsu and Murphy, 2004). This form of activity-dependent asynchronous release has been described previously in the hypothalamus (Kombian et al., 2000a); it manifests as a postsynaptic barrage of AMPA-mediated events lasting for several seconds in response to prolonged, high-frequency activation of glutamatergic afferents (Kombian et al., 2000a). It is not clear whether this short-term potentiation in the SON represents a summation of individual asynchronous epochs (such as those we have described in PVN) (Iremonger and Bains, 2007) or whether it requires the recruitment of other (unique) cellular mechanisms. Our observation that asynchronous release can be potentiated during short, physiologically relevant (Washburn et al., 2000) trains of stimuli suggests that it may share some features with previously described short-term potentiation in SON (Kombian et al., 2000a).

These unique properties raise the question of why these synapses in particular would exhibit asynchronous release. One plausible explanation is that since the rate of hormone release from MNC terminals is roughly proportional to their firing frequency (Leng et al., 1999), synaptic precision and timing are not essential features for successful operation of the magnocellular system. Instead, these cells are specialized to integrate inputs from several nuclei into a final "output" signal. Asynchronous glutamate release may provide a mechanism to amplify presynaptic activity and promote

prolonged spiking in response to temporally dispersed afferent inputs and may be a particularly effective way to ensure a faithful neuronal response during intense physiological demand; in effect it may serve as the ignition switch for a prolonged discharge in MNCs. Previous work showing that asynchronous release in recurrently connected networks of hippocampal neurons is necessary for bursting (Lau and Bi, 2005; Wyart et al., 2005) supports the idea that this form of transmission may be functionally important for translating synaptic signals into specific patterns of neuronal activity. Since neurons that provide excitatory input to MNCs exhibit bursts of activity (Washburn et al., 2000), our observations that a brief afferent discharge can increase postsynaptic activity for a prolonged period of time indicates that asynchronous release is an important component of signal transfer at these synapses. Second, the prolonged depolarization (and spiking) mediated by asynchronous release may facilitate the opening of postsynaptic NMDA receptors and voltage-gated Ca^{2+} channels. The resulting Ca^{2+} influx may promote both the dendritic release of peptides (Ludwig and Pittman, 2003) and the induction of synaptic plasticity (Panatier et al., 2006a).

Mechanisms responsible for asynchronous release in PVN

Action-potential-dependent neurotransmitter release relies on the opening of presynaptic voltage-gated Ca^{2+} channels. More specifically, the N-type and P/Q type channels have been implicated in neurotransmitter release in the vertebrate CNS (Lisman et al., 2007). The specific complement of Ca^{2+} channel subtypes may allow synapses to release transmitter with kinetically distinct profiles (Wu et al., 1999). In some brain regions, including the calyx of Held, the kinetics of transmitter release are developmentally regulated, mirroring the developmental changes in the expression of different Ca^{2+} channel subtypes (Fedchyshyn and Wang, 2005). Our observations indicate that asynchronous release in PVN, however, is present in both young (p22) and older (p44) animals suggesting that it is a conserved form of neurotransmission in this nucleus and is not sensitive to developmental regulation (Iremonger and Bains, 2007). Recent work in the hippocampus indicates that in addition to these possibilities, the mode of release may depend on the complement of Ca^{2+} channel subtypes expressed at the presynaptic nerve terminal (Hefft and Jonas, 2005). In particular, P/Q type Ca^{2+} channels may be important for synchronized transmitter release whereas N-type channels are necessary for asynchronous (Hefft and Jonas, 2005) or ectopic (Matsui and Jahr, 2004) release. This is consistent with the observation that N-type channels may be located a greater distance from the docked vesicle and thus their recruitment releases transmitter on a slower time scale (Wu et al., 1999). This is currently under investigation in our laboratory. In addition to different Ca^{2+} channels, asynchronous release may also be a consequence of a larger distance between the Ca^{2+} source and the Ca^{2+} sensor associated with the vesicle (Meinrenken et al., 2002; Fedchyshyn and Wang, 2005; Hefft and Jonas, 2005) or a poor Ca^{2+} buffering capacity in the presynaptic nerve terminal (Muller et al., 2007). Our observation that asynchronous release in particular is sensitive to low concentrations of the slow Ca^{2+} buffer, EGTA-AM (Iremonger and Bains, 2007) is consistent with any or all of these scenarios (Fig. 3). Additional mechanisms, such as a prolonged presynaptic Ca^{2+} transient (Atluri and Regehr, 1996) and the presence of different Ca^{2+} sensors (with different Ca^{2+} affinities) for asynchronous compared to synchronous release (Geppert et al., 1994; Nishiki and Augustine, 2004; Hui et al., 2005), also cannot be ruled out at this stage.

Evoked glutamate release and the regulation of MNC activity

Despite the obvious requirement for both synaptic and postsynaptic conductances in determining the firing patterns of MNCs (Nissen et al., 1995; Li and Hatton, 1996; Leng et al., 1999; Brown et al., 2004; Brown and Bourque, 2006), the interaction between these two factors has yet to be studied carefully in the PVN or SON. Summation of excitatory synaptic inputs is an obvious mechanism capable of evoking action potentials. The DAP which follows each action potential would then allow for maintenance of a plateau potential and repetitive spiking. Alternatively, prolonged glutamate release

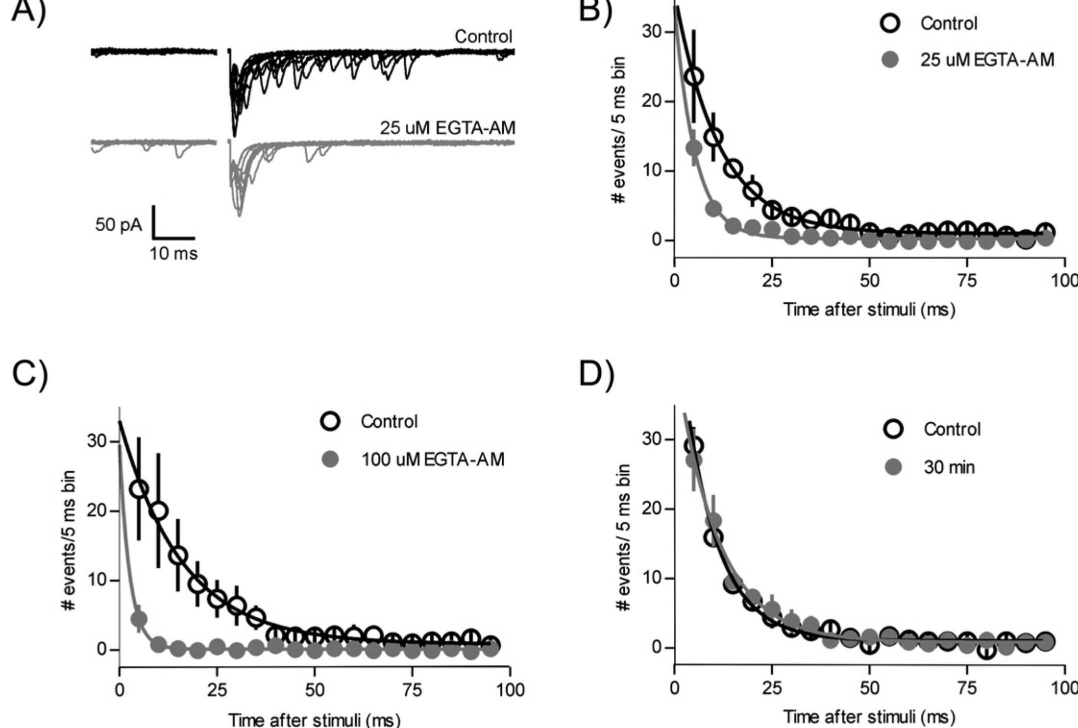

Fig. 3. Asynchronous release is dependent on slow rises in presynaptic Ca^{2+}. (A) Voltage-clamp traces from a single neuron show the effect of the membrane-permeable Ca^{2+} chelator EGTA-AM (25 μM, 15 min) on glutamate release. (B) The summary graph from five cells demonstrates that there is a preferential decrease of the asynchronous release component. (C) Increasing the concentration of EGTA-AM (100 μM) decreases asynchronous release to an even greater extent ($n = 6$). (D) Continuous whole-cell recording with no pharmacological manipulations shows no rundown of asynchronous release over the 30 min. Figure is adapted with permission from data in Iremonger and Bains (2007).

may also directly recruit DAPs via activation of NMDA receptors and Ca^{2+} permeable AMPA receptors. It has been shown by several groups that magnocellular neurons have functional NMDA receptors (Hu and Bourque, 1992; Bains and Ferguson, 1997, 1999) and that glial cells within the SON release d-Serine (Panatier et al., 2006b), the endogenous co-agonist of the NMDA receptor. Since it has been suggested that the DAP is mediated by a Ca^{2+}-sensitive cation channel (Ghamari-Langroudi and Bourque, 2002), the Ca^{2+} influx resulting from NMDA receptor activation may contribute to the plateau potential that allows phasic burst firing in AVP neurons. In addition, this NMDA receptor activation may even be critical for the release of dendritic dense core vesicles. For example, it is known that priming of dense core vesicles is Ca^{2+}-dependent as this can be achieved through application of thapsigargin or CPA, compounds that expel Ca^{2+} form internal stores (Ludwig et al., 2002) and that NMDA receptor activation can initiate dendritic Ca^{2+} spikes in MNCs (Bains and Ferguson, 1999). The Ca^{2+} influx resulting from NMDA receptor activation could alone, or in conjunction with Ca^{2+} induced Ca^{2+} release from internal stores, induce priming of dendritic dense core vesicle and their subsequent fusion. Although presently, we do not know if this indeed occurs, these are all testable ideas that will likely be answered within the next several years.

Conclusions

Glutamate synapses onto MNCs are geared to transmit signals for hundreds of milliseconds. This

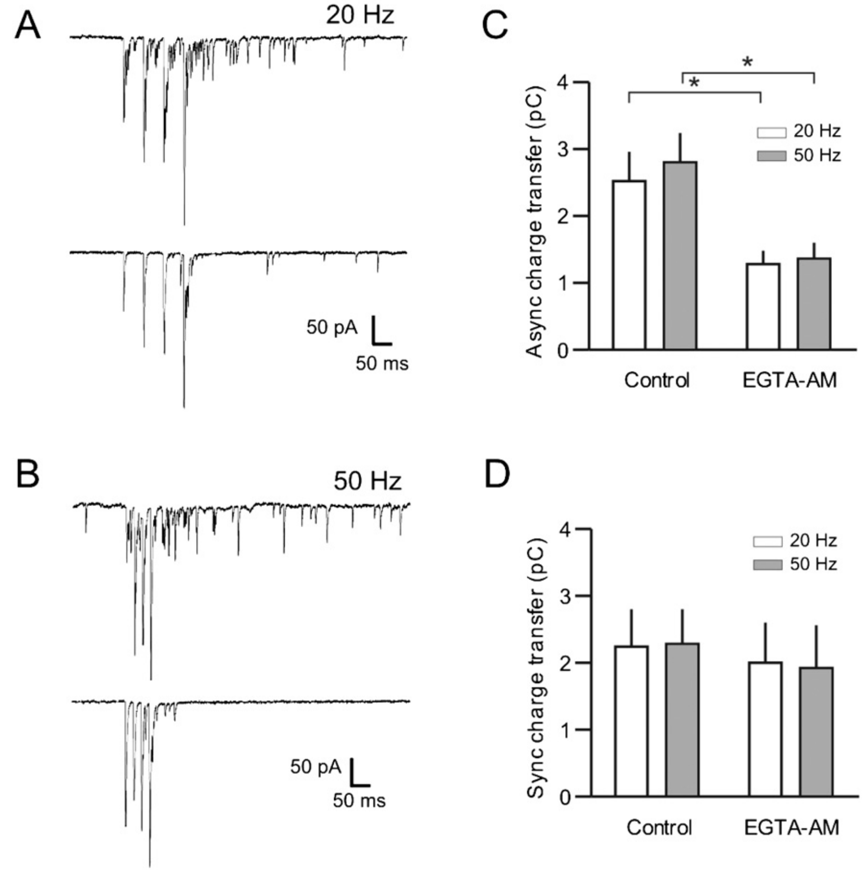

Fig. 4. Asynchronous release accumulates during short trains of afferent activity. (A, B) There is a facilitation of asynchronous release after short (four pulse) trains at 20 (A) and 50 Hz (B). The release after the end of the train is also sensitive to low concentrations of EGTA-AM (top traces are control and bottom traces are after application of 25 μM EGTA-AM). Stimulation artefacts have been removed for clarity. (C) Quantification of these data show that asynchronous charge transfer elicited by 20 and 50 Hz trains in control was 2.53 ± 0.44 and 2.79 ± 0.47 pC, respectively. After application of 25 μM EGTA-AM, asynchronous release was 1.28 ± 0.21 and 1.39 ± 0.20 pC for the 20 and 50 Hz trains, respectively ($^*p<0.05$; $n = 5$). (D) The synchronous component of release during the trains was not inhibited after EGTA-AM ($p>0.05$; $n = 5$). Figure is adapted with permission from data in Iremonger and Bains (2007).

extended temporal window can be further prolonged when synapses are repetitively recruited (Fig. 4). Under these conditions, the integration of these synaptic events by the MNCs may sufficiently depolarize the neuronal membrane and serve as an effective ‘ignition switch’ that activates the postsynaptic conductances necessary to sustain high-frequency discharges (Fig. 5). Asynchronous release may also be an ideal synaptic means by which peptides can be released from MNC dendrites. Finally, the asynchronous release discussed above indicates that glutamate synapses onto MNCs in PVN are capable of releasing multiple quanta. In some cases, these quanta may be released in an asynchronous fashion (as described here) or, if the right machinery is activated, multiple vesicles may be released simultaneously. For example, the recruitment of Ca^{2+} from intracellular stores by noradrenaline can synchronize the release of many quanta (multivesicular release) which may further increase postsynaptic excitability (Gordon and Bains, 2005).

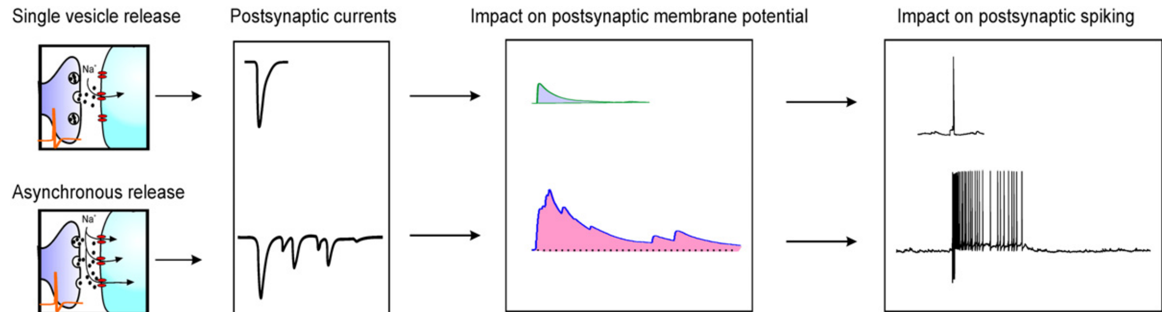

Fig. 5. The dynamics of release determines the effect of an EPSP on postsynaptic excitability. Cartoon showing fast and synchronized (top) or prolonged and desynchronized (bottom) release of glutamate in response to a single presynaptic action potential. In the top example, there is fast and transient depolarization of the cell that may evoke a single action potential. In the bottom example, asynchronous release results in a large and prolonged depolarization that is capable of eliciting a burst of action potentials and may promote regenerative firing via activation of DAPs. (See Color Plate 11.5 in color plate section.)

Acknowledgements

The research highlighted here was supported by an operating grant from the Canadian Institutes for Health Research (CIHR). J.S. Bains is an Alberta Heritage Foundation for Medical Research (AHFMR) Senior Scholar. K.J. Iremonger is supported by studentships from the AHFMR and the Heart and Stroke Foundation of Canada.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 12

Endogenous modulators of synaptic transmission: cannabinoid regulation in the supraoptic nucleus

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Abstract: The magnocellular neurons of the hypothalamic supraoptic nucleus (SON) are a major source of both systemic and central release of the neurohypophyseal peptides, oxytocin (OXT) and arginine–vasopressin (AVP). Both OXT and AVP are released from the somatodendritic compartment of magnocellular neurons and act within the SON to modulate the electrophysiological function of these cells. Cannabinoids (CBs) affect hormonal output and the SON may represent a neural substrate through which CBs exert specific physiological and behavioural effects. Dynamic modulation of synaptic inputs is a fundamental mechanism through which neuronal output is controlled. Dendritically released OXT acts on autoreceptors to generate endocannabinoids (eCBs) which modify both excitatory and inhibitory inputs to OXT neurons through actions on presynaptic CB receptors. As such, OXT and eCBs cooperate to shape the electrophysiological properties of magnocellular OXT neurons, regulating the physiological function of this nucleus. Further study of eCB signalling in the SON, including its interaction with AVP neurons, promises to extend our understanding of the synaptic regulation of SON physiological function.

Keywords: hypothalamus; oxytocin; magnocellular neurons; retrograde messengers

Introduction

The hypothalamic-neurohypophyseal system consists of two nuclei, the supraoptic nucleus (SON), situated lateral to the optic chiasm, and the paraventricular nucleus (PVN), on each side of the third ventricle. Magnocellular neurons in these nuclei synthesize either oxytocin (OXT) or arginine–vasopressin (AVP) and send axonal projections to the posterior pituitary. Neuronal activity stimulates release of the hormones into the blood, regulating a number of important physiological functions, most notably lactation/parturition (OXT; Neumann et al., 1993, 1994; Leng et al., 2005) and body–fluid homoeostasis (AVP; Landgraf et al., 1988; Leng et al., 1999). The somatodendritic regions of magnocellular neurons are also a major site of OXT and AVP release within the central nervous system (CNS) and contain many peptidergic large dense core vesicles (LDCVs). These vesicles release their contents in response to elevated calcium, which can be

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DOI: 10.1016/S0079-6123(08)00412-3

elevated independent of electrical activity in the neuron (Pow and Morris, 1989; Ludwig et al., 2002). It has been suggested that OXT and AVP may diffuse to affect remote brain areas that do not receive direct OXT/AVP innervation (Ludwig and Leng, 2006).

Central neuropeptide release impacts on systemic hormone output and the role of somatodendritic neuropeptide release in shaping electrical activity of magnocellular neurons has been an area of investigation for many years (Ludwig and Pittman, 2003). Although somatodendritic and axonal release often respond to the same stimuli, there is evidence that the release can be independently regulated from each compartment. For example, during lactation and parturition, levels of OXT increase within the SON before systemic levels rise (Neumann et al., 1993). This may be important because OXT neurons are excited by OXT itself and injection of OXT into the SON facilitates its own release (Moos et al., 1984; Yamashita et al., 1987). In vivo, central AVP, which is also found to be regulated by known stimuli (e.g. osmotic), acts within the SON to bring the spiking activity of AVP neurons to an intermediate level (Gouzenes et al., 1998) which maximizes the effectiveness of hormonal output at the neurohypophysis (Dutton and Dyball, 1979). AVP also facilitates its own release through action on AVP receptors within the nucleus (Wotjak et al., 1994).

Functional disparities between AVP and OXT neurons may reflect, to a degree, differing regulation by somatodendritically released neuropeptides (Oliet et al., 2007). Recent reports have highlighted the interaction of the endogenous cannabinoid (CB) system with central hormone release in the modulation of magnocellular neuron synaptic physiology (Hirasawa et al., 2004; Sabatier and Leng, 2006).

The cannabinoid system in the SON

Cooperative signalling of OXT and the cannabinoid system

Two subtypes of CB receptor, CB_1 and CB_2, have been identified and cloned (Matsuda et al., 1990; Munro et al., 1993) although there is evidence for further subtypes (Hajos et al., 2001). The CB_1 receptor is the predominant CNS subtype (Freund et al., 2003), whereas the CB_2 receptor is primarily expressed in immune cells, although it is also found in the CNS (Van Sickle et al., 2005). CB receptors are believed to be primarily associated with presynaptic terminals (Freund et al., 2003) and studies on recombinant receptors expressed in cultured neurons agree with in vitro reports (Katona et al., 1999; Egertova and Elphick, 2000) indicating the selective expression of CB_1 receptors at presynaptic sites (Leterrier et al., 2006; McDonald et al., 2007). Depolarization of the postsynaptic cell generates endocannabinoids (eCBs) which act retrogradely to inhibit subsequent neurotransmitter release; depending on whether the presynaptic input is excitatory or inhibitory, this phenomenon is termed depolarization-induced suppression of excitation or inhibition (DSE/DSI) respectively (Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001).

Dynamic modulation of synaptic inputs constitutes a fundamental mechanism through which neuronal output is regulated. A number of neuromodulatory transmitters postsynaptically influence, or presynaptically regulate excitatory and inhibitory inputs to SON magnocellular neurons; a non-exclusive list includes glutamate (Schrader and Tasker, 1997), noradrenaline (Wang et al., 1998), dopamine (Price and Pittman, 2001), acetylcholine (Li and Pan, 2001; Li et al., 2001; Hatton and Yang, 2002), histamine (Hatton and Yang, 2001), adenosine (Oliet and Poulain, 1999), nitric oxide (Stern and Ludwig, 2001; Gillard et al., 2007) and a number of peptides (e.g. Galanin, Papas and Bourque, 1997; Kozoriz et al., 2006; pituitary adenylate cyclase activating polypeptide (PACAP), Gillard et al., 2006).

OXT itself modulates excitatory neurotransmission onto magnocellular neurons through what were thought to be presynaptic receptors (Kombian et al., 1997; de Kock et al., 2003). However, OXT receptor expression in the SON is largely localized postsynaptically (Freund-Mercier et al., 1994; Adan et al., 1995). The link between OXT action and presynaptic glutamate release became apparent when it was subsequently found that exogenously

applied CB agonists also presynaptically inhibit neurotransmission in the SON (Hirasawa et al., 2004; Soya et al., 2005; Oliet et al., 2007). Immunoreactivity for the CB_1 receptor within the SON is consistent with a presynaptic localization, with electron microscopy confirming that expression of the CB_1 receptor is restricted to axons (Fig. 1). The apparent presynaptic action of OXT was blocked by a CB antagonist, suggesting that somatodendritically released OXT activates OXT autoreceptors and triggers eCB synthesis to presynaptically inhibit glutamatergic transmission (Fig. 2); DSE in this nucleus is also blocked by OXT antagonists (Hirasawa et al., 2004). Metabotropic regulation of eCB production is well documented; in the hippocampus, for example, muscarinic activation can potentiate, and may occlude, DSE (Varma et al., 2001; Straiker and Mackie, 2007). However, it appears that in the SON activation of OXT autoreceptors is a prerequisite for eCB release with depolarization alone being insufficient (Hirasawa et al., 2004).

The role of tonic eCB production in determining distinct functional characteristics of OXT and AVP neurons

There is evidence for a basal eCB tone in the SON (Di et al., 2005; Oliet et al., 2007). GABAergic neurotransmission onto OXT neurons is tonically inhibited by eCBs. Since this tonic eCB generation is dependent on constitutive OXT release, this underlies the distinct characteristics of inhibitory inputs onto OXT and AVP neurons. The phenotype of the postsynaptic cell determines the strength of presynaptic GABAergic inputs. The tonic, concerted action of OXT and eCBs selectively maintains a low probability of release at GABAergic synapses onto OXT cells, allowing facilitation of GABAergic transmission under repetitive stimulation and the effective termination of bursting (Oliet et al., 2007). Furthermore, GABAergic inputs appear to be important in determining an irregularity in basal firing of OXT neurons (Li et al., 2007), which is believed to

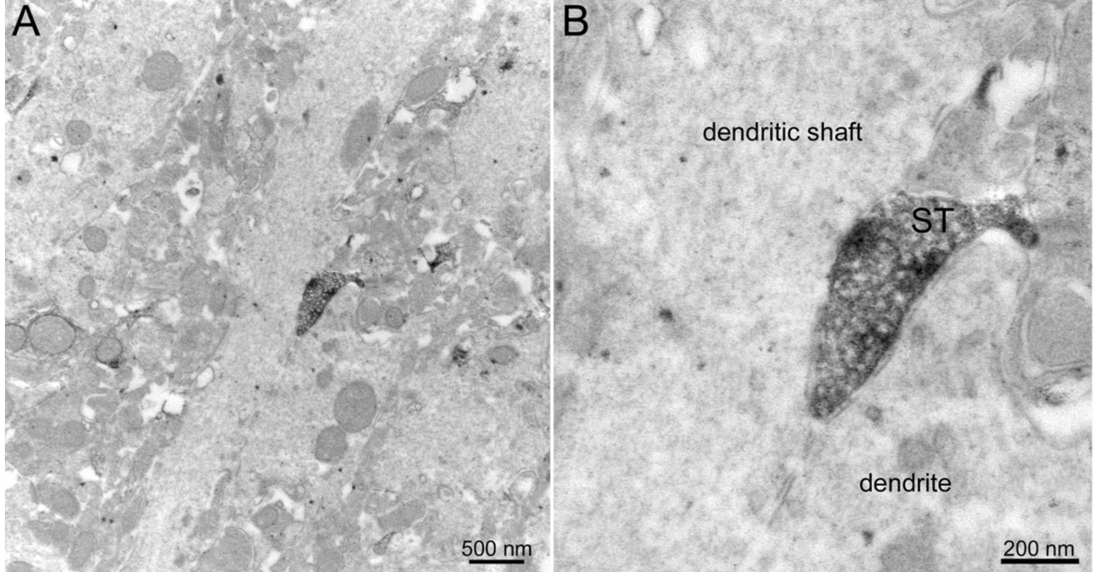

Fig. 1. The subcellular distribution of the CB_1 receptor is restricted to presynaptic processes in the SON. (A, B) Electron micrograph showing a synaptic terminal (ST) labelled for CB_1 (dark deposit), contacting a dendritic shaft. Note that the expression of the CB_1 receptor within the SON is restricted to presynaptic processes. (B) Magnified area of micrograph shown in A.

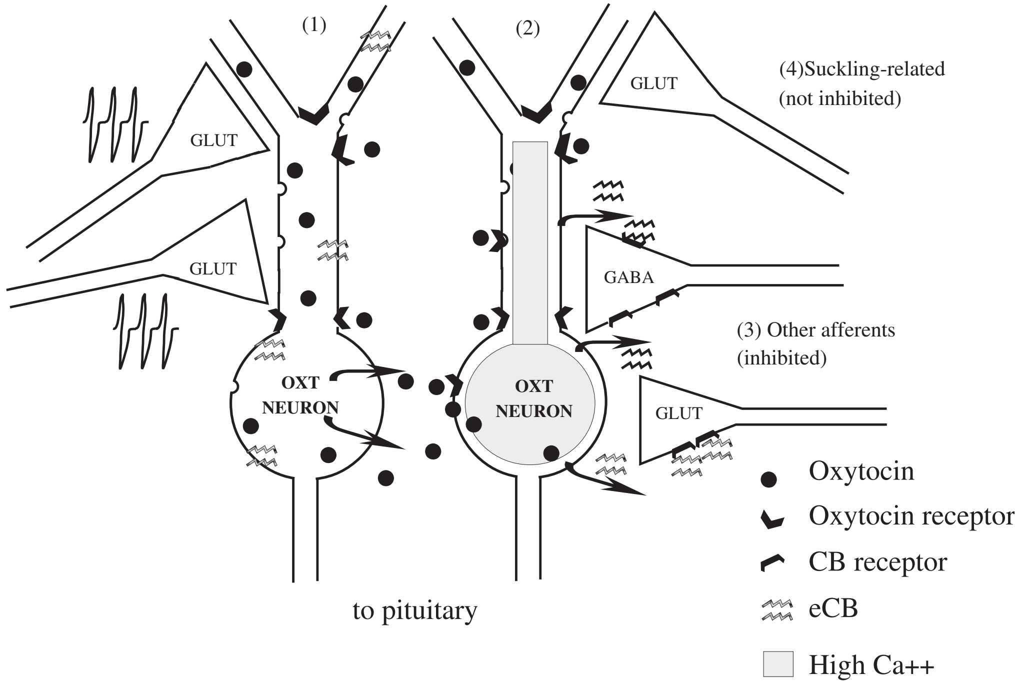

Fig. 2. Retrograde signalling by eCBs in the SON. Schematic illustrating the proposed mechanism of cooperative signalling by OXT and eCBs in the modulation of synaptic inputs to magnocellular neurons in the SON. (1) Excitatory stimulation and depolarization of OXT magnocellular neurons causes the somatodendritic release of OXT which acts on OXT autoreceptors, (2) leading to calcium release from stores and the generation of eCBs. OXT may diffuse to neighbouring OXT neurons to initiate the temporal and spatial spread of CB signalling. (3) Acting at presynaptic CB receptors, eCBs inhibit both glutamatergic and GABAergic afferents onto magnocellular neurons. (4) Specific afferents, lacking CB_1 receptors and perhaps, for example, related to suckling, may be unaffected by eCBs.

facilitate their probability of bursting (Moos et al., 2004). Therefore, it is likely that the tonic regulation of these inputs by basal eCB production contributes to maintaining this conducive state.

Role of cannabinoids in the independent control of central and systemic OXT release

Neurotransmitters which elevate intracellular calcium can potentially stimulate somatodendritic neuropeptide release. As such, somatodendritic OXT/AVP release is not dependent on action potential firing and can be stimulated, in the absence of electrical activity, without a corresponding rise in systemic levels (Ludwig et al., 2002). It has recently been reported that somatodendritic release of OXT is stimulated by α-melanocyte-stimulating hormone whilst concurrent inhibition of excitatory input onto OXT cells through eCB release, which may be secondary to somatodendritic OXT release, reduces peripheral secretion of the hormone (Sabatier, 2006). Interestingly, administration of either OXT or α-melanocyte-stimulating hormone within the CNS leads to similar effects on appetite and sexual behaviour. It is proposed that it is the increase of OXT within the SON induced by α-melanocyte-stimulating hormone that mediates these behavioural outcomes.

Cannabinoids and AVP neuron function

Studies examining the role of the CB system in magnocellular neuron electrophysiology have primarily concentrated on the relationship between eCB and OXT signalling. AVP preferentially inhibits excitatory over inhibitory inputs to

magnocellular neurons (Kombian et al., 2000) with the locus and mechanism of this effect remaining undetermined. Cannabis usage is associated with diuresis, indicative of reduced systemic AVP release, consistent with CBs selectively inhibiting excitatory inputs to AVP neurons. Although both exogenously applied CB agonists and postsynaptic depolarization presynaptically inhibit excitatory and inhibitory transmission regardless of magnocellular phenotype (Hirasawa et al., 2004; Soya et al., 2005), Oliet et al. (2007) report that tonic CB production selectively inhibits GABAergic inputs onto OXT neurons.

Central AVP release in response to dehydration is dependent on PACAP receptor activation. Administration of PACAP is associated with decreased glutamate release within the SON. The similarity of this response profile to that of OXT action on OXT neurons raises the possibility that eCBs may be released and mediate the reduction in glutamate release (Gillard et al., 2006). However, nitric oxide, which stimulates central whilst reducing systemic AVP release, stimulates glutamate release within the SON (Gillard et al., 2007). Thus, perhaps it is possible to override eCB tone with other secretagogues.

Future perspectives

It has been suggested that the functional organization of the eCB system in the SON may be unique compared with what has been reported for other areas of the CNS. In magnocellular neurons of the PVN, glucocorticoid-induced eCB synthesis is dependent on G_s-stimulated cAMP production (Malcher-Lopes et al., 2006); perhaps protein kinase A-activated, calcium-insensitive isoforms of CB synthesis enzymes are expressed in magnocellular neurons. Another unresolved question is why eCBs do not appear to be generated by depolarization-induced calcium influx alone. Calcium released from ryanodine sensitive stores has been shown to play a role in eCB production (Isokawa and Alger, 2006). In fact, in an autaptic preparation of hippocampal neurons, DSE was found to require calcium release from stores (Straiker and Mackie, 2005). Coupling eCB generation selectively to calcium release from stores rather than calcium-influx resulting from depolarization could result from a tighter control of calcium-induced calcium release in OXT neurons. In the SON, OXT actions on autoreceptors might conceivably increase the sensitivity of the CB system, amplifying eCB signalling. Furthermore, it is conceivable that OXT may diffuse further than eCBs, and thus, allow eCB action on many cells to co-ordinate activity, transforming a local effect to a global one.

The further study of eCB signalling in the SON promises to illuminate many more aspects of the physiological function of this nucleus. What is still lacking is an understanding of how the eCB system works in a functional sense. We are still faced with the conundrum that OXT is *facilitory* in promoting OXT release from neurohypophyseal axons during lactation and delivery of pups. Yet our electrophysiological studies indicate an inhibitory action, mediated by eCBs. In resolving this apparent contradiction, it may be appropriate to recall that magnocellular neurons receive many different inputs, in addition to the glutamatergic and GABAergic ones discussed here. Some of these, such as the noradrenergic, histaminergic, cholinergic and various peptidergic afferents may be resistant to eCB retrograde inhibition (due to lack of presynaptic CB receptors). Thus signals important in lactation and birth may influence the OXT neuron, whereas extraneous inputs from other sources (mediating stress inputs for example) will be suppressed at this time (Fig. 2). Alternatively, retrograde signalling may be important during lactation to regulate the activity level of the neuron to enable efficient peptide release from the neurohypophysis. It is also possible that there may be gender-specific effects of OXT since in vivo studies have largely been conducted on lactating rats whereas much of the in vitro work uses male rats.

To resolve the role of eCBs in regulating magnocellular activity in vivo, it may be useful to consider that the CB_1-knockout mouse is able to deliver pups and to provide them with milk (Fride et al., 2003 and unpublished observations). Although it has been reported that CB_1-knockout pups are somewhat compromised in feeding during the first couple of days after delivery, this is

thought to be due to a problem in the oro-facial musculature functioning of the pups (Fride et al., 2005). If the pattern and delivery of milk by the mother is normal in these mice (something that has not been investigated properly to the best of our knowledge), this would argue that the eCBs are not of major importance in the regulation of OXT neuronal activity during lactation. Future studies might also be profitably directed at understanding how these animals respond to other stimuli known to activate magnocellular neurons.

Abbreviations

AVP	arginine–vasopressin
CB	cannabinoid
CNS	central nervous system
DSE/ DSI	depolarization-induced suppression of excitation or inhibition
eCBs	endocannabinoids
LDCV	large dense core vesicle
OXT	oxytocin
PACAP	pituitary adenylate cyclase activating polypeptide
PVN	paraventricular nucleus
SON	supraoptic nucleus

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 13

Oxytocin and appetite

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Abstract: Oxytocin has potent central effects on feeding behaviour, as well as on social and sexual behaviours, and one likely substrate for its anorectic effect is the ventromedial nucleus of the hypothalamus. This nucleus expresses a high density of oxytocin receptors, but contains very few oxytocin-containing fibres, hence it is a likely target of 'neurohormonal' actions of oxytocin, including possibly oxytocin released from the dendrites of magnocellular oxytocin neurones. As oxytocin release from dendrites is regulated independent of electrical activity and of secretion from the neurohypophysis, exactly how this release is regulated by metabolic and reproduction-related signals remains to be established fully. Intriguingly though, it looks as though this central release of oxytocin from magnocellular neurons might be instrumental in a fundamental shift in motivational behaviour — switching behaviour from being driven by the need to find and consume food, to the need to reproduce.

Keywords: supraoptic nucleus; ventromedial hypothalamus; prolactin-releasing peptide; dendritic release

Introduction

Classically, feeding is regulated by an alternation between 'hunger' signals, which activate specific hunger centres in the hypothalamus, and 'satiety signals', which activate specific satiety centres. The hunger and satiety centres interact with each other, and are modulated according to the energy stores and momentary metabolic requirements; these are signalled by both circulating factors (such as plasma concentrations of leptin, insulin and ghrelin) and by neurally mediated signals, some of which arise from the gastro-intestinal tract. How these signals are processed depends on the animal's internal state, reflecting varying motivational drives, and is sensitive to diverse environmental cues, including those arising from photoperiod.

A striking example of the complex motivational effects on appetite regulation is the mutually exclusive nature of feeding-related behaviours and sexual behaviours. Put most simply, for all animals, the most important drives are to eat and to reproduce. As it is important that each is satisfied efficiently and effectively, animals commit their behaviour to achieving either sex or food, rather than risk being both hungry and frustrated by failing to give their undivided attention to either goal. As a well-fed animal may be better able to compete for a mate and then to copulate with sustained enthusiasm, the natural course of events is that the first priority of hungry animals is to eat, and when sated they

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DOI: 10.1016/S0079-6123(08)00413-5

turn to sex. This seems to be achieved by a reciprocal regulation of sexual and ingestive behaviours, in which, for mammals hypothalamic oxytocin appears to play an interesting and possibly important part.

The oxytocin and vasopressin peptide lineages arose by a gene duplication that probably happened early in vertebrate evolution. Most invertebrates express a single peptide of this family, expressed in multifunctional neurons that regulate various aspects of reproduction, electrolyte balance and energy intake and utilization. In mammals, oxytocin neurons include magnocellular neurons that project to the neurohypophysis and which regulate lactation and parturition by oxytocin released into the peripheral circulation, and parvocellular oxytocin neurons that project to the brainstem and spinal cord, and which are involved in both control of food intake and control of penile erection. However, magnocellular neurons also release very large amounts of oxytocin within the hypothalamus, where oxytocin is reciprocally involved in the motivation for sexual behaviour (in both males and females) and in appetite.

The ventromedial nucleus of the hypothalamus (VMH) is a likely focus of some of these central motivational effects of oxytocin. It has a density of oxytocin receptors that is one of the highest in the brain, yet appears virtually devoid of innervation by oxytocin-containing fibres. This nucleus is a likely target of oxytocin released from magnocellular neurons, either by oxytocin released in an activity-dependent way from the axons of magnocellular neurons that circumnavigate the VMH, passing through areas penetrated by the dendrites of VMH neurons, or by the oxytocin that is released in massive amounts by the more distant dendrites of the magnocellular neurons. We end this review by pointing out that the massive amounts of oxytocin contained within each neurosecretory vesicle, and the relative rarity of release events, mean that central release of oxytocin cannot plausibly be organized as a signal with temporal and anatomical specificity, instead, oxytocin release in the brain is intrinsically and inevitably a hormone-like signal.

Oxytocin and oxytocin-like peptides throughout evolution

Oxytocin and vasopressin are produced by separate genes, but the genes are so closely related that they are clearly derived by duplication of a common ancestor gene at some point in our evolutionary history. At first sight, oxytocin appears to be a quintessentially mammalian hormone; released from the neurohypophysis in response to suckling, it appears to be absolutely indispensable for suckling-induced milk let-down, as well as being important for parturition (see Russell et al., 2003). All eutherian mammals make oxytocin; most marsupials (metatheria) make mesotocin, which is important in both lactation and parturition in marsupials as in eutherian mammals. Mesotocin differs from oxytocin by a single amino acid, is equally potent at the oxytocin receptor and is thought to reflect a neutral mutation.

However, it is not just mammals that make an oxytocin-like peptide; all vertebrates except for the cyclostomes (hagfishes and lamprey), have two neurohypophysial hormones, one closely related to vasopressin and one to oxytocin. In birds, mesotocin replaces oxytocin, acting through the oxytocin receptor, and intracerebroventricular injections of oxytocin cause a dose-dependent decrease in food intake, feeding time and pecking frequency (Jonaidi et al, 2003).

By contrast, most invertebrates have just one oxytocin/vasopressin related peptide, with the curious exception of the mollusc cephalopods, which have both an oxytocin-like peptide (cephalotocin) and a vasopressin-like peptide (octopressin) (Takuwa-Kuroda et al., 2003). Thus oxytocin and vasopressin evolved via duplication of an ancestral gene at least 400 million years ago, before the evolution of the fishes, but possibly even earlier (hagfishes and lampreys evolved over 550 million years ago).

The arginine vasotocin/isotocin neuroendocrine system in fishes

The arginine vasotocin/isotocin neuroendocrine system regulates many aspects of fish physiology

and behaviour, including circadian and seasonal biology, responses to stress, metabolism, reproduction, cardiovascular function and osmoregulation. Isotocin and vasotocin, the fish homologues of oxytocin and vasopressin, are similarly expressed in magnocellular neurons that project to the neurohypophysis, and are best known for their roles in oviposition (egg-laying) and osmoregulation. The neuron-specific expression of the oxytocin and vasopressin gene families, and the mechanisms mediating this cell-specificity, evolved before the divergence of the fish and mammalian lineages, as was elegantly demonstrated when Gilligan et al. (2003) generated a transgenic mouse bearing overlapping Fugu cosmids that contained the isotocin and/or vasotocin genes: the Fugu isotocin and vasotocin genes were found to be expressed specifically in the mouse oxytocin and vasopressin neurons, respectively.

Both peptides have behavioural effects as well as these hormone-like actions. In territorial fish species, vasotocin has a role in aggressive behaviour analogous to that described for vasopressin in the prairie vole (see Young et al., 2005). Isotocin also has roles in reproductive behaviour, and there is evidence for its involvement in behaviours that depend critically on social interaction. In the teleost fish *Lythrypnus dalli*, when a dominant male is removed from the social group, the dominant female will change sex to male, and this change is associated with a marked loss of isotocin immunoreactivity in the preoptic area of the brain (Black et al., 2004). In eels, isotocin and vasotocin are also involved in ingestive behaviour, as fibres containing these peptides innervate the esophageal sphincter muscles (Watanabe et al., 2007); here the two peptides are mutually antagonistic, and it is thought that alternating drives from the two neuronal populations promote sequential muscle contraction and relaxation during ingestion.

In cytclostomes, vasotocin (the presumed direct common ancestor of vasopressin and oxytocin) is expressed in neurons of the preoptic nucleus of the hypothalamus. These project to the neurohypophysis, but also to other brain areas, notably to the reticular formation (Nozaki and Gorbman, 1983). The physiological role of vasotocin in cytclostomes is unclear, but it seems to have a role in energy metabolism as well as in osmoregulation (John et al., 1977).

Roles of oxytocin/vasopressin like peptides in invertebrates

In modern invertebrates, peptides related to oxytocin or vasopressin are expressed in neurons and are involved in reproduction and osmoregulation, but also in feeding and energy utilization. Seven such vasopressin/oxytocin-related peptides have so far been isolated in different invertebrate phyla. Annetocin was first characterized in the earthworm *Eisenia foetida*, and is mainly expressed in anterior neurons, in the subesophageal ganglia, and in areas involved in the regulation of reproduction (Satake et al., 1999). Injections of annetocin induce egg-laying behaviour in earthworms and in leeches, but also produce a concomitant loss of body-weight. As annetocin also stimulates gut contraction in the earthworm (Ukena et al., 1995), it may have a role in energy intake/utilization.

Conopressin is abundantly expressed in the central nervous system of most gastropods, and is also present in the penis nerve and in fibres that innervate the vas deferens; conopressin induces muscular contractions of the vas deferens and inhibits central neurons that control female reproductive behaviour. In the simultaneous hermaphrodite snail *Lymnaea stagnalis*, conopressin is present in most neurons of the anterior lobe of the right cerebral ganglion, most of which project to the male copulatory organ (the penile complex). During copulation, when the male copulatory organ is completely everted, these neurons are electrically active, and as stimulation of these neurons can induce eversion (De Boer et al., 1997), it seems that these neurons control the snail equivalent of penile erection.

Interactions between feeding and sex

Feeding and sexual behaviour are mutually exclusive goal-orientated behaviours. For example,

consider the invertebrate *C. elegans*. To mate, the adult male must first find an appropriate partner (a hermaphrodite) and will wander about its environment to locate one. If an adult male is left isolated on a food source it will eventually leave the food to search for a hermaphrodite. This mate-searching behaviour is called 'leaving behaviour' (Lipton et al., 2004). In food-deprived animals, this 'leaving' behaviour is reduced, suggesting that it is responsive to nutritional status, and that a lower nutritional status will decrease sex drive. Interestingly, this mate-searching behaviour is under the control of a single gene, and this gene is regulated by an insulin-response element.

When an appropriate partner is found, mating begins when the tail of the male contacts his mate. Several subsequent behaviours are needed for successful copulation. One crucial step is the insertion of the male's two copulatory spicules into its mate, where they must remain in position until sperm transfer. Each spicule is attached to retractor muscles that maintain the spicule in the male's tail, and protractor muscles that eject the spicule during mating and keep it inside the mate until copulation is completed. The male spicule muscles are regulated by pharyngeal neurons. If the pharynx is pumping abnormally, for example in the absence of food, the pharyngeal neurons suppress the protraction of the spicules. Garcia and Sternberg (2003) found that a mutation of a gene coding for an ERG- like K^+ channels will induce spontaneous protraction of spicules in the absence of any hermaphrodite cues; this spontaneous spicule protraction is food dependent. During contact with its mate, the pharynx in males will eventually stop pumping but it will continue in males where the pharyngeal neurons have been ablated. Importantly, this suggests that the same neurons that regulate pharyngeal activity (and thus feeding) also regulate mating.

Although feeding behaviour varies between herbivorous and carnivorous gastropods, in all gastropod species, feeding behaviours depend on the internal state of the animal, its current environment, and its past experience. The herbivorous *Aplysia* spends most of its time either feeding or mating. Simply removing food increases mating behaviour (Susswein, 1984), but isolation from potential mates inhibits feeding (Botzer et al., 1991). Conversely, pheromones from potential mates can facilitate feeding behaviour (Nedvetzki et al., 1998), suggesting that, if a potential mate is present, the animal will eat vigorously until sated, allowing it to spend the rest of the day mating, without interference from distracting hunger pangs.

Regulation of feeding in mammals

Thus, throughout evolution, there is a close reciprocal organization of appetites for food and sex. In mammals, central actions of oxytocin inhibit feeding and stimulate sexual arousal, suggesting that oxytocin may play an important role in orchestrating these behaviours. In mammals, energy intake and utilization is regulated by several interconnected populations of neurons in the hypothalamus and caudal brainstem. These express many different neuropeptides that have orexigenic or anorexigenic effects when injected centrally. Among the most important, are neurons in the arcuate nucleus that make neuropeptide Y (NPY), a potent orexigen; these are activated by circulating ghrelin (secreted from the empty stomach), and inhibited by circulating leptin (secreted from adipocytes in proportion to the body's stores of fat). Within the arcuate nucleus, NPY neurons innervate pro-opiomelanocortin (POMC) neurons (see Cone, 2005); this inhibitory projection is mediated mainly by GABA which is co-expressed with NPY. The POMC neurons in turn produce α-melanocyte-stimulating hormone (α-MSH), which has potent anorectic effects mediated mainly by hypothalamic MC4 receptors. α-MSH appears to be essential for normal regulation of food intake, as mutations of either the POMC gene or of the MC4 receptor are conspicuously associated with hyperphagia and adiposity. However, α-MSH is equally involved in sexual arousal, being a potent promoter of penile erections, again through its actions at MC4 receptors. The NPY neurons also synthesize agouti-related protein (AgRP), an endogenous antagonist at the MC4 receptors. The POMC neurons are activated by leptin and inhibited

by ghrelin, both directly and indirectly via the NPY/AgRP neurons. Both POMC neurons and NPY/AgRP neurons project densely to the paraventricular nucleus of the hypothalamus (PVN), which has several roles in the regulation of metabolism, including: (i) through its control of the sympathetic nervous system; (ii) through regulation of thyroid stimulating hormone secretion; (iii) through the regulation of ACTH secretion and production of the catabolic glucocorticoid hormones and (iv) through descending projections to the gastric regulatory centres of the caudal brainstem.

The arcuate neuronal populations are regulated not only by circulating factors, but also by ascending signals from the brainstem that mediate signals from the gut. Among the ascending projections, noradrenergic neurons of the A2 cell group in the nucleus tractus solitarii (NTS) are activated by gastric distension and by peripheral CCK, a satiety-signalling peptide secreted from the gut in the presence of food. Several peptides are co-expressed in subpopulations of the noradrenergic neurons, and these neurons are also functionally diverse, but other peptide-expressing neurons in the NTS that do not express noradrenaline also project to the hypothalamus, and apparently also carry feeding-related signals that arise from the gut. Thus several peptide messengers act in the hypothalamus to mediate signals from the gastrointestinal tract; one of the most important being prolactin-releasing peptide (PrRP), which we will talk more of later. Blood-borne signals related to nutrient status are also detected by neurons in the area postrema; a circumventricular organ adjacent to the NTS that is outside the blood–brain barrier, and which is densely interconnected with the NTS.

Oxytocin and feeding behaviour

Within the brain, oxytocin is released from centrally projecting parvocellular neurons of the PVN, and from the soma and dendrites of magnocellular neurons of the PVN and SON. The magnocellular oxytocin regions of both nuclei are innervated by fibres containing α-MSH, and the oxytocin cells densely express mRNA for the MC4 receptor (see Sabatier, 2006). Parvocellular regions of the PVN are innervated by both NPY- and α-MSH-containing fibres from the arcuate nucleus.

Oxytocin-containing nerve endings are present in many brain areas, but are especially dense in the brainstem and spinal cord. The first suggestion that oxytocin might be involved in regulating feeding behaviour, came from Verbalis and colleagues (e.g. Olson et al., 1991a, b) who noted that increases in plasma oxytocin concentrations are generally accompanied by reductions in food intake. Peripheral injections of oxytocin have no effect on food intake, but i.c.v. injections of oxytocin or oxytocin agonist potently inhibit feeding, and these effects are prevented by an oxytocin antagonist (e.g. Arletti et al., 1990; Lokrantz et al., 1997; see Sabatier, 2006 and Douglas et al., 2007).

There is clear evidence of a role of parvocellular oxytocin neurons in feeding. These neurons densely project to the NTS, where they innervate NTS neurons that are activated by CCK during feeding, supporting the hypothesis that oxytocin potentiates the inhibitory action of CCK on meal size, and leptin modulates this action of oxytocin (e.g. Blevins et al., 2003, 2004).

However, there is also evidence that magnocellular oxytocin neurons are involved in regulating appetite. Fos expression is increased in the supraoptic nucleus (SON) in rats after food intake (Johnstone et al., 2006); Fos expression and oxytocin secretion are also strongly activated by gastric distension or by systemic injections of CCK (see Sabatier, 2006), and fasting reduces the expression of nitric oxide synthesis in magnocellular neurons — an effect reversed by leptin treatment (Isse et al., 1999). As nitric oxide synthase is expressed in both oxytocin cells and vasopressin cells and is produced in an activity-dependent manner, this probably reflects an inhibitory effect of fasting on magnocellular neuronal activity, though this has not been studied directly. Interestingly, α-MSH inhibits the electrical activity of magnocellular oxytocin neurons, and so reduces oxytocin secretion into the systemic circulation, but potently stimulates oxytocin release from neuronal dendrites. This central release of oxytocin is mediated by MC4 receptors, and the release is a consequence of peptide-induced

mobilization of intracellular calcium stores. Thus it seems that α-MSH selectively stimulates central, rather than peripheral oxytocin release from magnocellular neurons (Sabatier et al., 2003; Sabatier and Leng, 2006). The dendrites of magnocellular neurons are by far the largest store of oxytocin in the brain. Each neuron has typically two or three large dendrites, together accounting for about 80% of the total cell volume of a magnocellular neuron — and, in the SON, the dendrites contain >85% of the total oxytocin content of the SON. The hypothalamus contains about 9000 magnocellular oxytocin neurons; each of their dendrites contains several thousand of the large neurosecretory vesicles in which oxytocin is packaged, and each vesicle contains about 85,000 molecules of oxytocin. These dendrites release large amounts of oxytocin semi-independently of the electrical activity of the cell bodies; although some peptides can trigger release of oxytocin from the dendrites, electrical activity of the cells does not normally evoke release, but some peptides can 'prime' the dendritic stores of peptide, making them available for release in response to electrical activity (Ludwig and Leng, 2006).

Oxytocin and sexual behaviour

Both peripheral and central oxytocin are involved in erectile function in rats (see Leng et al., 2005). During copulation, oxytocin is secreted from the neurohypophysis into the peripheral circulation with a large pulse released at ejaculation, and receptors in the rat penis seem to mediate contractility in vivo. In addition, oxytocin is released within the brain (Waldherr and Neumann, 2007) and has major effects on sexual arousal; i.c.v. injections induce penile erection and yawning in rats, whereas injections of oxytocin antagonist decreased mounting and intromission frequencies and abolished ejaculation. Parvocellular oxytocin neurons project to the spinal cord, and this is one important site at which oxytocin controls penile erection, but intrahypothalamic actions are also important; injection of oxytocin in the PVN induces penile erection, and lesions of the PVN impair oxytocin-induced penile erection. Central oxytocin also stimulates female sexual behaviour by increasing sexual receptivity and facilitating lordosis, thought to be at least in part by actions at the VMH.

The timing of central and peripheral release of oxytocin during mating is not clear, as this complex behaviour involves several distinct phases from motivation to ejaculation. Peripheral oxytocin is needed for the consummatory phase of sexual behaviour, but central oxytocin might also be important for the motivational phase; interestingly, it seems that the effects of α-MSH on sexual behaviour might be mediated in part by its actions upon magnocellular oxytocin neurons to induce dendritic oxytocin release (Caquineau et al., 2006).

Appetite in oxytocin-deficient mice

Oxytocin-deficient mice have normal body weight, but there are several abnormalities of ingestive behaviour in these mice. In rats, stimuli that increase oxytocin release peripherally are generally accompanied by a reduction in food intake — dehydration for example, which is a potent stimulus for oxytocin secretion as well as for vasopressin secretion, is accompanied by a profound depression of appetite. Such dehydration-induced anorexia is attenuated in oxytocin-deficient mice (Rinaman et al., 2005), while consumption of solutions that contain NaCl is enhanced (Puryear et al., 2001; Amico et al., 2003; Vollmer et al., 2006). This is not solely attributable to any specific effect on sodium appetite, as oxytocin-deficient mice will also overconsume palatable sucrose solutions (Miedlar et al., 2007), and both sweet and non-sweet carbohydrate solutions (Sclafani et al., 2007).

While mice lacking oxytocin appear generally normal in body weight, male mice lacking the oxytocin receptor (oxytocin receptor-KO mice) have an overt, though mild, late-onset obesity, being slightly heavier than wild-type mice. However, we have found no significant difference in the daily total food intake of wild-type and OTR-KO mice. As some parvocellular oxytocin neurons in the PVN project polysynaptically to brown adipose tissue (Oldfield et al., 2002), it is possible that oxytocin is involved in peripheral regulation of energy metabolism. We found that,

when OTR-KO mice are placed in a cold environment (5°C), their body temperature decreases more than wild-type mice, so a reduced energy consumption might contribute to the late-onset obesity.

Because stressful stimuli activate both magnocellular and parvocellular oxytocin neurons in the hypothalamus, we analyzed the effects of stress on food intake in male OTR-KO mice. Immobilization for 1 h reduced the amounts of food eaten over the following 24 h in both wild-type and OTR-KO mice, but there was no significant difference between the groups, suggesting that oxytocin receptors are not necessary to decrease food intake following stressful stimuli.

The total amounts of daily food intake in home cages are not significantly different between wild-type and OTR-KO mice, but OTR-KO mice eat significantly more at each meal than wild-type mice, although the meal frequency per day is not significantly different. CCK-A receptor KO mice show a very similar phenotype (Bi et al., 2004); as CCK is an important peripheral satiety-signalling peptide, it is thus possible that oxytocin receptors are involved in satiety signalling within the brain.

Although the precise neuronal source of the oxytocin that is involved in food intake remains to be determined conclusively, the magnocellular oxytocin neurons in the hypothalamus are the major source of oxytocin within the hypothalamus. Magnocellular oxytocin neurons receive a strong noradrenaline/PrRP projection from the medulla oblongata, so we examined whether this projection is involved in the activation of oxytocin neurons following stressful stimuli (Onaka, 2004) and CCK administration. We found that conditioned fear stimuli activated PrRP neurons identified retrogradely as projecting to the SON, and that neutralizing endogenous PrRP by monoclonal antibodies attenuated oxytocin secretion from the pituitary in response to conditioned fear; the increase in plasma oxytocin concentrations induced by conditioned fear was also blocked in PrRP-KO mice. In addition, application of PrRP induced somato-dendritic oxytocin release from the isolated SON in vitro. We also examined the role of PrRP following food intake. Food intake activated PrRP neurons in the medulla oblongata. Oxytocin secretion following CCK administration was attenuated in PrRP-KO mice, suggesting that PrRP projections play a role in activating magnocellular oxytocin neurons following food intake.

We thus examined food intake in PrRP-KO mice in more detail. PrRP-KO mice showed late-onset obesity and hyperphagia. The size of meals eaten, but not their frequency, was increased in PrRP-KO mice; essentially the PrRP-KO mice differ from wild-type mice in eating more during meals, but less between meals. Meal size is normally limited by acute signals arising from the distended stomach, and CCK is thought to be an important mediator of this, acting via CCK-A receptors on afferent gastric vagal nerve endings. CCK-induced anorexia was blocked in PrRP-KO mice, consistent with the conclusion that PrRP relays satiety signalling within the brain (Bechtold and Luckman, 2006). Furthermore, expression of PrRP mRNA is reduced in hyperphagic conditions such as lactation in rats (Bechtold and Luckman, 2007), and in streptozotocin-induced diabetic or Zucker diabetic rats (Mera et al., 2007).

The ventromedial nucleus of the hypothalamus (VMH)

The above data suggest that PrRP–oxytocin system is important for satiety signalling. The major brainstem centre for satiety signalling is the NTS; however, in the hypothalamus, the major site appears to be the VMH.

The VMH is mostly known for its involvement in the regulation of sexual and feeding behaviours in the rat, although it also regulates blood pressure and pain pathways. It is a heterogeneous nucleus that can be subdivided into dorsomedial, central and ventrolateral regions based on the expression of transcription factors, receptors and neuropeptides (McClellan et al., 2006). For example, brain-derived neurotrophic factor (BDNF) and pituitary adenylate cyclase-activating polypeptide (PACAP) are expressed in discrete populations of neurons that are scattered throughout the nucleus, while nitric oxide synthase, somatostatin and enkephalin are expressed in neurons found mainly in the ventrolateral region of the nucleus.

The ventrolateral region of the VMH is thought to be mainly responsible for the facilitation of lordosis behaviour in female rats, and this is thought to be associated with the presence there of neurons that express estrogen receptors (Pfaff and Keiner, 1973; Pfaff and Sakuma, 1979). The dorsal VMH has been known as a satiety centre since 1940, when it was first reported that rats with lesions of the VMH display a 'voracious' appetite. One interesting subpopulation of VMH neurons, which delineates the more dorsal and central regions, uniquely expresses the nuclear receptor steroidogenic factor-1 (SF1). Deletion of SF1 results in abnormal VMH development and obesity in mice (Majdic et al., 2002), and mutations of SF1 are linked to obesity and type-2 diabetes in humans (Liu et al., 2006). Leptin excites SF1 neurons in vitro, and the actions of leptin in the VMH help to resist diet-induced obesity (Dhillon et al., 2006). Another population of VMH neurons is involved in glucose homoeostasis, and contains neurons that respond to changes in extracellular glucose (Song et al., 2001), these glucoreceptor neurons are also activated by leptin in vivo (Shiraishi et al., 2000).

The VMH projects to the anterior hypothalamus, the medial preoptic area and the PVN, and has the major extrahypothalamic projections to the amygdala, the bed nucleus of the stria terminalis and the periaqueductal grey. The ventrolateral VMH, which is particularly associated with sexual behaviour, projects to thalamic nuclei, the retrochiasmatic area, the raphe and the dorsomedial hypothalamus, whereas the dorsomedial region, which is specifically important for appetite, projects to the bed nucleus of the stria terminalis, the nucleus accumbens and the medial prefrontal cortex. The medial VMH send strong excitatory inputs to proopiomelanocortin (POMC) neurons in the arcuate nucleus; this connection is dynamically regulated by nutritional state, as it is weakened by fasting (Sternson et al., 2005).

In the rat brain, the VMH is among the regions that contains the highest densities of oxytocin binding sites as shown by autoradiography (Freund-Mercier et al., 1987; Tribollet et al., 1988; Kremarik et al., 1995; Bale et al., 2001) and oxytocin receptor mRNA expression, as shown by in situ hybridization (Yoshimura et al., 1993; Bale et al., 1995; Bale and Dorsa, 1995), with particularly intense labelling in the ventrolateral region of the VMH. A similar distribution of oxytocin binding sites was found in guinea-pig (Tribollet et al., 1992), and vole (Young et al., 1996).

In the female rat, oxytocin receptors in the VMH are regulated by ovarian hormones estradiol and progesterone, and their activation by oxytocin facilitates lordosis (Schumacher et al., 1989). Estradiol treatment induces a four-fold increase in the number of oxytocin binding sites in the VMH (de Kloet et al., 1986; Coirini et al., 1989), and increases expression of oxytocin receptor mRNA (Bale and Dorsa, 1995). In estrogen-primed rats, progesterone also induced an expansion of the area occupied by oxytocin receptors into the periphery of the VMH, where dendrites of VMH neurons are found (Schumacher et al., 1989). Following focal electrolytic lesions of the VMH, oxytocin receptor binding is reduced by over 80% in the area surrounding the nucleus, indicating that most of the oxytocin receptors there are located on fibres originating within the VMH, most likely on the dendrites of VMH neurons which extend into the extranuclear region (Johnson et al., 1991). However, in the same study, the estradiol-induced increase in oxytocin binding was higher within the VMH than in the extranuclear region, suggesting that the cells bodies of VMH neurons are the most important site for the expression of functional oxytocin receptors.

The presence of functional oxytocin receptors in the VMH has been confirmed by electrophysiological studies. In extracellular recordings of single VMH neurons in hypothalamic slices, Kow and Pfaff (1986) reported that 58% of VMH neurons did not respond to oxytocin, 28% were excited and 14% were inhibited. In a similar study, these authors found that 94% of the oxytocin-responding neurons recorded in the ventrolateral region of the VMH responded with an excitation of their electrical activity, and this excitatory response was enhanced by estradiol pre-treatment (Kow et al., 1991). Oxytocin applied on slices of guinea-pig hypothalamus excited about half of the VMH neurons tested, whereas none were inhibited (Inenaga et al., 1991).

There is still very little known about the electrophysiological behaviour of these neurons in vivo, and in particular, about the effects of appetite- and sex-related peptides such as oxytocin, on their firing pattern. We performed extracellular recordings of single VMH neurons in vivo in urethane-anaesthetized male rats and studied the effects of injecting 1–10 ng of oxytocin (in 1–2 μl) directly into the third ventricle. We found that 51% of 65 VMH neurons tested were excited by oxytocin (Fig. 1), while 21% were inhibited and 28% were not affected.

The abundance of functional oxytocin receptors on cell bodies and dendrites of VMH neurons contrasts strikingly with the absence of oxytocin-containing fibres and synapses innervating the nucleus. The VMH contains very few fibres that show any immunoreactivity for either oxytocin or vasopressin (Caldwell et al., 1988; Jirikowski et al., 1988; Schumacher et al., 1989; Fig. 2), and it is not known whether the few oxytocin fibres there are 'stray' axons or dendrites of magnocellular neurons, or come from parvocellular neurons of the PVN. By marked contrast to the virtual absence of oxytocin fibres in the VMH, the region ventrolateral to the VMH is densely permeated by both vasopressin- and oxytocin-containing axons of magnocellular neurons on their way from the PVN and SON to the neurohypophysis (Daniels and Flanagan-Cato, 2000), and by the axons of parvocellular neurosecretory neurons on their way to the median eminence. The magnocellular neuronal axons do not have any collateral branches in this region, so give rise to no conventional nerve endings and synapses. This does not however mean that no oxytocin is released from these axons; the large dense-cored vesicles can be released from any part

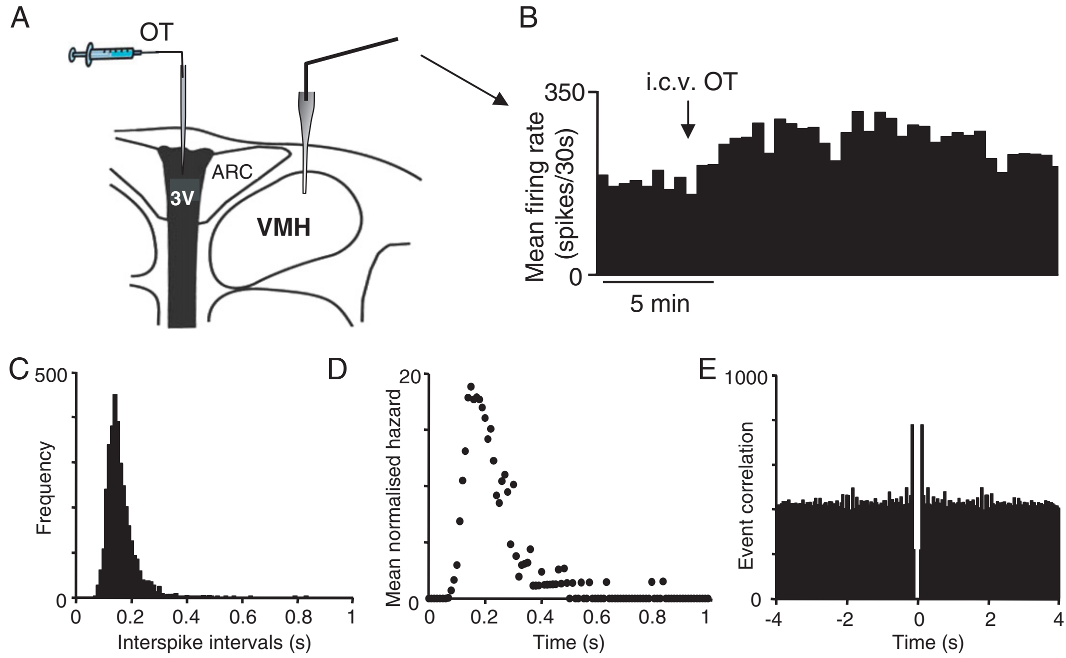

Fig. 1. (A) The ventral approach for electrophysiological recordings of VMH neurons in vivo. In urethane-anaesthetized rats, the ventral surface of the brain is exposed transpharyngeally; a glass electrode is placed in the VMH for extracellular recordings of single VMH neurons, and an i.c.v. cannula is implanted into the third ventricle for central injection of oxytocin. (B) In vivo recording of the mean firing rate in a single VMH neuron. Note the increase in firing rate after i.c.v. injection of 10 ng oxytocin. (C) Interspike interval distribution of the neuron shown in (B) constructed in 10-ms bins from 5 min of stationary spontaneous discharge activity. (D) hazard function constructed from the interspike interval distribution shown on (B). The hazard function was normalized to the total hazard over the first 500 ms. (E) Event correlation histogram for the neuron recorded in (B). 3 V, third ventricle; ARC, arcuate nucleus; VMH, ventromedial hypothalamus.

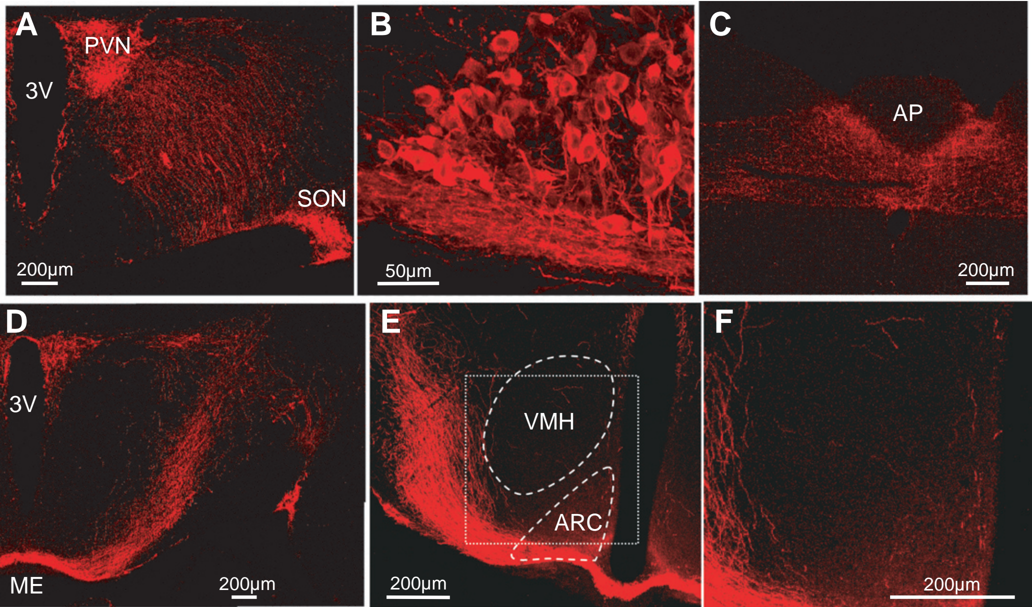

Fig. 2. Immunohistochemical detection of oxytocin in hypothalamic sections of the rat brain. (A) Coronal hemi-section of the anterior hypothalamus showing oxytocin cells in the PVN and the SON, and the network of oxytocin-containing axons. (B) Coronal section of the SON showing oxytocin cells and their dendritic processes. Note that oxytocin-containing dendrites are organized in a dense network on the ventral surface of the brain. (C) Coronal section of the brainstem showing its innervation by oxytocin-containing fibres from parvocellular cells in the PVN. (D) Coronal hemi-section of the posterior hypothalamus showing the network of oxytocin-containing axons from magnocellular neurons; these axons enter the internal zone of the median eminence on their way to the neurohypophysis. (E) Coronal hemi-section of the posterior hypothalamus containing the VMH and the arcuate nucleus. Note the network of oxytocin-containing axons running through the median eminence. (F) Zoom in on the ventromedial hypothalamus from section shown in (E) (dotted square). Note the virtually complete absence of oxytocin-containing fibres in the VMH. 3 V, third ventricle; PVN, paraventricular nucleus; SON, supraoptic nucleus; AP, area postrema; ME, median eminence; ARC, arcuate nucleus; VMH, ventromedial nucleus of the hypothalamus. (See Color Plate 13.2 in color plate section.)

of a neuron in which they are present, even including undilated axons, the likelihood of a vesicle being released appears to depend solely on how close it is to the cell plasma membrane. Axons contain relatively few vesicles that are close to the plasma membrane, and far fewer in total than are contained in dendrites, but nevertheless might be a functionally significant source of oxytocin in the region adjacent to the VMH. So far, there has been no direct evidence of any projection to or synaptic innervation of VMH neurons by oxytocin neurons from the parvocellular region of the PVN.

Peptides and behaviour

Peptides are important messengers in the central nervous system. Most neurons secrete at least one of more than a hundred different peptides, each of which can act at specific, high-affinity G-protein coupled receptors, expressed specifically on subpopulations of neurons — indeed many of the peptides have several distinct receptors, expressed differentially, and not only on other neurons but also on glial cells and on blood vessels (see Leng and Ludwig, 2006). These receptors have nanomolar affinity for their endogenous ligands, and are diversely coupled to intracellular signalling systems with diverse consequences: peptides can modulate gene expression, synaptogenesis and local blood flow, as well as modulate the excitability of neuronal targets in complex and subtle ways. Yet few neurons secrete only peptides; most communicate with each other using one of a mere handful of conventional neurotransmitters — most make either the

excitatory transmitter glutamate or the inhibitory transmitter GABA. Indeed, for conventional information transmission in the brain it seems surprising that they should need any more than these two; neurotransmitters scarcely escape from the synaptic clefts into which they are released, so they can be spatially precise in their actions without any need for a target-specific chemical recognition system. Faithful transmission of information encoded in spike trains requires that the spikes lead to a rapid release of transmitter that has short lasting effects confined to specific target cells. This fidelity is assured because the small vesicles that contain the conventional transmitter are specifically targeted to synapses. The synapses are specialized in containing the exocytotic machinery that enables activity-dependent transmitter release, and the environment of the synapse contains the enzymes and pumps needed to clear transmitter from the synapse rapidly after release. The receptors for neurotransmitters do not have particularly high affinity for their ligands; indeed, if they did, they would be continually activated by the inevitable spillover of transmitter released onto adjacent neurons. Neurotransmitter receptors have affinities in the micromolar range; making the target cell sensitive only to the peak concentrations achieved within the synapse immediately after synaptic transmitter release. This helps to ensure that transmitter release results in an excitatory or inhibitory postsynaptic potential that lasts for just a few milliseconds, allowing the target cell time to be ready again for the next spike and the next signal.

Neuroendocrine neurons have, time and again, given us powerful metaphors for understanding neuronal function, but the success of those metaphors has misled us perhaps into neglecting some of those things that make neuroendocrine systems important and special in their own right. In thinking that other neurons in the brain are merely less splendid versions of our own neurons, we have perhaps forgotten that, in part, our neuroendocrine neurons are so very splendid because of the things by which they are different. We have been both arrogant and strangely insecure, arrogant in thinking of our neurons because they are exemplars *par excellence* for all neurons, insecure in forgetting that these are *important* neurons, that would be just as important if they were nothing like any other neuron in the brain.

The neurohypophysis has long been a model system for studying stimulus–secretion coupling, as it contains only axons and their nerve terminals, terminals that contain a vast amount of their secretory products, oxytocin and vasopressin. Like conventional neurotransmitters, the peptides are released by calcium-dependent exocytosis, triggered when action potentials invade the nerve endings. However, the amount released notably depends on the *patterning* of electrical activity, an insight that has proved of general significance, as it became apparent that patterning of spike activity was a key determinant of information transfer wherever it has been studied. It thus becomes critically important to consider exactly what electrical signals are effective in releasing peptide-containing vesicles from fibres in the brain. This is very difficult to assess, but we can start by considering very carefully exactly what message is needed to release the neurohypophysial peptides oxytocin and vasopressin from their nerve endings in the neurohypophysis, because this we can calculate exactly.

Each of the 18,000 axons (from 9000 oxytocin cells and 9000 vasopressin cells) that enters the neurohypophysis has $\sim$2000 nerve endings; each ending contains $\sim$220 large, dense-cored vesicles that contain oxytocin or vasopressin, and several hundred larger swellings, each containing $\sim$1000 vesicles (Morris, 1976a, b). Each vesicle contains $\sim$85,000 molecules of peptide (Nordmann and Morris, 1984), giving a total content of $\sim$1–2 μg of each peptide in the neurohypophysis. This is a vast store of peptide, and it must be. Conventional neurotransmitters can be recycled after release at a synapse, or can be made at the synapse, but peptides must be made in the cell nucleus and transported down the axon, and this takes time. So a large reserve of peptide must be retained in the neurohypophysis in case of a strong and unanticipated demand for secretion. The stores, though large, are not excessive, because even 1 day of

dehydration will significantly deplete the neural lobe content of vasopressin and oxytocin, both of which are secreted in response to a rise in plasma osmolarity.

We can establish how much is secreted by measuring the plasma concentrations of oxytocin and vasopressin; we know their half-life in the blood, so we can infer the secretory rate. We also know what the spike activity is like in oxytocin and vasopressin neurons, because of extensive recordings made by many labs. So we can ask, *how many vesicles are released, on average, from a nerve ending when it is invaded by a spike?*

In a normally hydrated, conscious rat, the plasma concentration of vasopressin is ~ 1 pg/ml. The plasma volume of a rat is not more than about 20 ml, but the distribution volume for vasopressin will be greater than this, as peripheral blood vessels are fenestrated, allowing the vasopressin to diffuse into extracellular space. However, for a 300 g rat, the total volume of extracellular fluid is <100 ml, so, at any one time there is <100 pg of vasopressin in the periphery. The half-life of vasopressin in rat plasma is ~ 5 min, so to maintain this content, 50 pg must be secreted every 5 min (10 pg/min; 6×10^9 molecules/min). As each vesicle contains 85,000 molecules, basal levels of vasopressin are maintained by the secretion of 7×10^4 vesicles/min. As there are 9000 magnocellular vasopressin cells, each with ~ 2000 nerve endings in the neurohypophysis, one vesicle is released every 25 min or so from each nerve ending.

Under urethane anaesthesia, vasopressin concentrations are ~ 10 pg/ml, needing cells to release one vesicle every 2.5 min or so. The mean firing rate of vasopressin cells in urethane anaesthetized rats is 3–4 spikes/s, so to release one vesicle from one nerve ending takes (on average) about 500 spikes. After acute osmotic stimuli, vasopressin secretion can be elevated to >100 pg/ml. The maximum mean sustained rate at which vasopressin cells can fire is ~ 10 spikes/s, and if the neurohypophysis is stimulated electrically at this mean frequency, the resulting plasma concentration is ~ 200 pg/ml. Again, this means that about one vesicle is released from each nerve ending for every 500 spikes or so.

The nerve endings of magnocellular neurons are packed with neurosecretory vesicles at a far higher density than seen in any peptide-containing nerve endings in the brain. *Yet even under the most intense stimulation, vesicle release from neurosecretory nerve endings is relatively rare. It takes several hundred spikes to cause just one vesicle to be released at any given nerve ending.*

Thus vesicle release is such a rare event in these nerve endings, so what about in the brain? Large dense-cored vesicles are found in synapses, but relatively few of them, far fewer than in the neurohypophysial nerve endings. Small synaptic vesicles are specifically targeted to synapses, but the large vesicles are distributed throughout the cytoplasm of neurons, and indeed can be released from the soma, the dendrites, even from undilated axons, they seem to need no specialized release sites. Instead they are mainly released from where they mainly are, and they are mainly not in synapses but in *dendrites*, because, for most neurons, about 80% of the cell volume is contained within its dendrites.

One SON of the rat contains about 1000 oxytocin cells and about 2000 vasopressin cells. Each cell has two or three dendrites, and each dendrite contains about 11,000 large dense-cored vesicles (see Pow and Morris, 1989). Compared to the neurohypophysis, this is a puny amount, but these cells are the richest peptide-containing cells in the brain. Consider one dendrite with its 11,000 vesicles. If release here occurs at proportionately the same rate as from nerve endings, then physiological release rates from a dendrite will be about 1–50 vesicles/min.

So what concentrations should we expect to be achieved by this? There are two important unknowns, the distribution volume and the clearance rate. The distribution volume in the acute phase is the extracellular space into which the oxytocin might diffuse. The volume of extracellular space in the brain is $<10\%$ of tissue volume, the volume of the rat hypothalamus is about 60 μl, so the extracellular fluid volume is <6 μl. If one vesicle is secreted from each of 9000 neurons, this is a total of 7.65×10^8 molecules of oxytocin or vasopressin — enough to provide a physiological concentration of 10 pM even if

diluted in a volume of over 100 μl. In short, vesicle release from individual nerve endings in the neurohypophysis is a rare event, even under conditions of intense stimulation, but rare events are enough because the vesicles contain so much. In the brain, release of oxytocin-containing vesicles is likely to be at least as rare from individual nerve endings. In the brainstem, the density of innervation is so great that parvocellular neurons provide a substantial local source of oxytocin, but in the hypothalamus and forebrain, it seems likely that the major source of oxytocin is the oxytocin that is released from magnocellular neuronal dendrites that reaches quite distant targets by volume transmission — diffusion aided by the bulk flow of extracellular fluid. As oxytocin release from dendrites is regulated independently of electrical activity and of secretion from the neurohypophysis, exactly how this release is regulated by metabolic and reproduction-related signals remains to be established fully. Intriguingly though, it looks as though this central release of oxytocin from magnocellular neurons might be instrumental in a fundamental shift in motivational behaviour — switching behaviour from being driven by the need to find and consume food, to the need to reproduce.

Acknowledgements

This work was supported by the Royal Society of Edinburgh, the BBSRC and the EU.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 14

Neural mechanisms underlying the milk ejection burst and reflex

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Abstract: One of the more interesting and complex phenomena involving neurohypophysial hormones is the milk ejection reflex and the events surrounding it. Accordingly, many investigations over the years have taken up the challenge of elucidating its myriad aspects. Much has been learned from in vivo preparations about the sequence of events that so regularly occurs: important priming by maternal behaviours, the intermittent rhythms, gating of bursting, synchrony of the oxytocin (OXT) neuronal bursts emitted intermittently in response to the continuous suckling of the young and the factors that influence the amplitude of the bursts/milk ejections (e.g. number of suckling pups). In vivo electrophysiological studies are constrained by the infeasibility of routinely recording transmembrane events and, therefore, cannot offer detailed membrane and/or synaptic analyses. Recent studies have developed an in vitro model of OXT neuronal bursting that has allowed more mechanistic analyses of these bursts as well as factors involved in their generation and structure. Here we review many of the cellular and molecular mechanisms that have been shown to underlie the milk ejection bursts, as revealed by in vitro analyses.

Keywords: astroglia; brain slices; electrophysiology; G-protein-coupled receptors; intracellular signalling cascades; oxytocin; oxytocin receptors; supraoptic nucleus

Introduction

For mammals, successful nurturing of the offspring is crucial for the survival of the individual and the species. Nursing of the young depends on two interrelated processes: milk production and milk ejection. Prolactin, an adenohypophysial hormone that induces the synthesis and secretion of milk by the mammary glands, is associated with milk production. Oxytocin (OXT), a hypothalamo-neurohypophysial hormone, acts to evoke contraction of the myoepithelial cells of the mammary glands to eject the stored milk. This action of OXT occurs during suckling by complex neuro-humoral mechanisms involving firing pattern conversion from tonic to intermittent burst discharges in hypothalamic OXT neurons.

Prior to nursing and the onset of milk ejections, however, there is a sequence of stereotyped maternal behaviours engaged in by the typical mother rat that prepares herself and her young for the bout of suckling. When the pups are only a few days old, the set of maternal behaviours prior to suckling includes pup retrieval to one locale in the nest, sniffing the individual pups, ano-genital licking to express urine from the pups' bladders, and assuming a crouching position over the grouped pups to allow them to

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DOI: 10.1016/S0079-6123(08)00414-7

begin nursing. It has long been thought that the strong sensory stimuli (particularly olfactory and vomeronasal) accompanying this sequence aids in priming relevant areas of the dam's neuroendocrine hypothalamus for milk ejection (Fahrbach and Pfaff, 1982). Varied evidence supports this idea, including morphological changes directly attributable to the maternal behaviours and altered functional properties that prepare the system for efficient peptide release (Salm et al., 1988; Modney et al., 1990; Ludwig and Leng, 2006). Indeed, there are direct projections to the neurons of the supraoptic nucleus (SON) from the main and accessory olfactory bulbs (Smithson et al., 1989, 1992) that convey olfactory- and vomeronasal-organ-derived information monosynaptically to OXT and arginine vasopressin (AVP) neurons (Hatton and Yang, 1989; Yang et al., 1995). Stimulation of the olfactory tract for 10 min significantly increased dye coupling between SON neurons (Hatton and Yang, 1989, 1990). A similar effect was obtained by exposure to pups (Modney et al., 1990). These excitatory pathways are almost certainly involved in the priming of OXT neurons that takes place immediately prior to suckling and milk ejections.

Ejection of milk in response to suckling is achieved via a classical neuroendocrine process, called the milk ejection reflex (MER) (Wakerley et al., 1994). Five links comprise the sensory–motor integrative process of the MER: (1) sensing the pups' (in the case of rats) suckling at the nipples, (2) conduction of neural impulses along the afferent pathway mediated by mammary nerves via dorsal root ganglia, (3) relay stations and integration in the spinal cord and brain, (4) conduction along efferent pathways via neural stalk/neurohypophysis (NS/NH) and blood circulation and (5) the receptors/effectors on mammary gland myoepithelium. Stimulation of tactile receptors at the nipples generates sensory impulses that are transmitted from nipples to course up the spinal cord and then to brain structures that influence or control OXT neuronal activity. Somewhere along the way, the suckling messages appear to be organized into "synchronizing signals" through close interactions of these structures with OXT neurons. These signals effect the simultaneous activation of virtually all OXT neurons. Synchronized bursts of a large pool of OXT neurons, but not individual neuronal bursts, lead to a bolus release of OXT from axon terminals in the NH, which first enters fenestrated capillaries there, and then the general circulation. Through the blood circulation, OXT is carried to the mammary tissue, where it causes contraction of the myoepithelial cells in the walls of the lactiferous ducts, sinuses and breast alveoli, leading to a sharp rise in intramammary pressure and the expulsion of milk from the teat. In humans, milk expulsions are often conditioned to the sound or smell of the infant, evoking milk-letdown even before the baby begins to suckle the breast, i.e. before the tactile stimulus of suckling begins (McNeilly et al., 1983).

Structural considerations: the SON

OXT neurons, generally intermixed to greater and lesser degrees with AVP neurons, are located in several magnocellular nuclei in the hypothalamus, including the SON, magnocellular division of the paraventricular (mPVN) nuclei, periventricular cell groups and several small accessory nuclei (Crowley and Armstrong, 1992). An overwhelming majority of these OXT neurons, especially those in the SON, projects parent axons to the NH via the NS. SON neurons, both OXT and AVP, tend to be bipolar and to have single dorsally projecting dendrites that give rise to the parent axons (Fig. 1). More abundant dendritic trees project ventrally into the ventral glial lamina (VGL), where they extend rostrocaudally among the resident astrocytes (Hatton, 1990). It is from these ventrally projecting SON dendrites that Pow and Morris (1989), using the tannic acid method and electron microscopy, first demonstrated stimulated vesicular release of OXT and AVP in vitro. Many subsequent in vivo studies have corroborated this phenomenon of intranuclear peptide release under physiological conditions (Neumann et al., 1993; Ludwig et al., 1994; Ludwig and Leng, 2006). Evidence for OXT release within the SON and its facilitatory effect on its own release was already available to add credibility to this developing story (Moos et al., 1984). Together these studies strongly foretold the existence of OXT receptors (OXTRs) in and around the SON.

Astrocytic cell bodies dominate the VGL, from which they project processes dorsally between

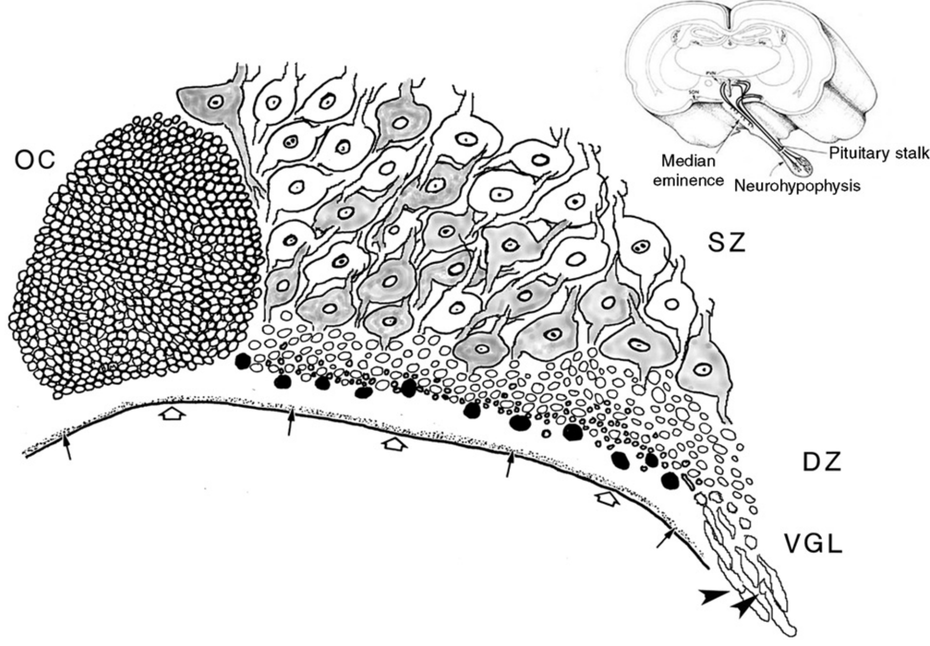

Fig. 1. Diagram of rat SON and the pial-glial limitans in coronal plane (right side of the brain). Profiles representing neuronal somata are in the somatic zone (SZ) lateral to the myelinated fibres of the optic chiasm (OC). Shaded profiles represent vasopressin- and clear profiles represent OXT- positive neurons. Dendrites projecting dorsally and ventrally from the somata have been truncated for simplicity, as have the axons arising from the dorsally projecting dendrites. Ventral to the SZ are the parallel-projecting dendrites (shaded and unshaded small ovoids), depicted in cross section, and constituting the dendritic zone (DZ). Elongated profiles at lower right are ventrolaterally projecting SON dendrites, which receive olfactory system inputs (filled arrowheads). Mingling with only the most ventral dendrites are the nuclei (larger filled profiles) of astroglial cells whose ventrally projecting processes fill the clear space between the basal lamina (small arrows) and the most ventral dendrites. These astroglial cell bodies and processes constitute the ventral glial lamina (VGL). Dorsally projecting processes from these glia fill most of the space not occupied by the somata and dendrites. Open arrows indicate pia mater. Inset: Diagram of the neurohypophysial system.

adjacent SON somata and dendrites, and ventrally to form an interface between neuronal membranes and the basal lamina lining the pial surface. Collaterals of magnocellular axons have been described in regions both dorsal and dorsolateral to the SON (Mason et al., 1984) and near the perifornical neurons lateral to the mPVN (Hatton et al., 1985). These collaterals, which appear to be glutamatergic as well as peptidergic (Meeker et al., 1991; Ponzio et al., 2006), may play crucial roles in the regulation of burst rhythms by delivering burst messages to other neurons in the local circuits (Boudaba and Tasker, 2006). During pregnancy and lactation, OXT neurons and SON astrocytes are highly plastic, both morphologically (Perlmutter et al., 1984; Langle et al., 2003) and functionally (Oliet et al., 2004), facilitating burst generation and synchronization. Compared to OXT neurons in the mPVN and accessory nuclei, those in the SON have been more extensively studied, as they are more abundant, more accessible and more amenable to identification. Thus, much of the research relating to mechanisms underlying neural bases of the MER has involved studies of OXT neurons in the SON.

Functional studies of milk ejection bursts and the MER

In vivo analyses

OXT neurons, recorded extracellularly in either unanaesthetized or anaesthetized rats, display milk ejection-associated burst firing patterns (Fig. 2),

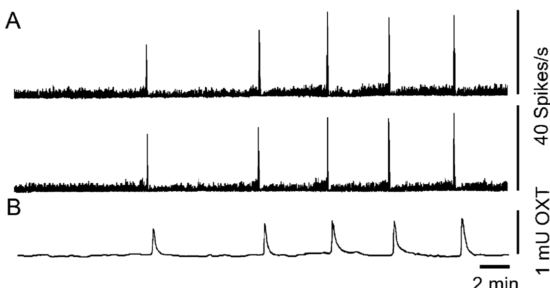

Fig. 2. In vivo bursts of supraoptic OXT neurons during suckling. (A) Simultaneous rate-meter records of OXT neuronal activity from two SONs. Two OXT neurons show simultaneous bursts shortly preceding intramammary pressure rises (B) as compared to that produced by 1 mU of OXT (scale bar). Adapted with permission from Wang and Hatton (2004).

consisting of transient (2–6 s) high-frequency (16–80 Hz) discharges of action potentials that recur every 2–15 min of continuous suckling (Wakerley and Lincoln, 1973; Summerlee and Lincoln, 1981). These synchronized bursts precede the expulsion of milk by ~10–12 s, the time it takes for the bolus of OXT, released in the NH, to reach and act on the mammary tissue. An interesting feature of OXT neuronal burst patterns, in addition to their intermittency in response to continuous suckling and their synchrony, is that the number of action potentials in a burst (burst amplitude) is related to the number of suckling pups. The more pups suckled, the higher the burst amplitude (Lincoln and Wakerley, 1975; Wang et al., 1996b). Also, early in a series of MERs, the amplitudes of subsequent bursts increase gradually and interburst intervals shorten, as more OXT neurons are recruited into the pool of bursting cells (Moos and Richard, 1988). As suckling progresses, virtually all of the OXT neurons located in the SON and mPVN, burst synchronously (Belin et al., 1984). Intervals >2 min between bursts are apparently required to maintain full mammary responsiveness, as more frequent release of OXT evoked by NH stimulation (e.g. 1/min) led to a decline in the amplitude of milk ejections (Summerlee and Lincoln, 1981).

The findings of in vivo analyses of bursts during nursing and of the MER have led to the postulation of three major mechanisms: summation, gating and synchronization (see Leng et al., 1999 for review). Summation is presumably achieved via accumulation of suckling signals and interactions between bilateral hypothalamic structures, and relates to the burst amplitudes being positively correlated with the number of suckling pups. Some sort of gating mechanism is presumed to account for the sudden occurrence of the bursts on a relatively stable rate of background firing, with a threshold level of activation being required to open the gate. It is still unclear just how continuous suckling signals are converted into intermittent burst discharges of OXT neurons. Since synchronization among the OXT neurons is both within each magnocellular nucleus and between the various nuclei, two (or more) separate processes may be involved in the synchronization mechanism. Synchronous elevations in excitability, leading to synchronous bursting among local groups of OXT neurons within the SON or mPVN may be mediated by any or all of the following: local circuit synaptic and/or astrocytic release of glutamate, dendritic release of OXT acting on OXT autoreceptors, dendritic release of glutamate from OXT neurons, and dendrodendritic gap junctional communication among neighbouring OXT cells. Gating demands that the intranuclear synchronization is under the control of internuclear synchronizing machinery. Internuclear synchrony appears to be achieved by chemical synaptic circuitry with some elements residing in the perinuclear areas, the posterior hypothalamus (Wang et al., 1995, 1996a, 1997) and the medulla (Moos et al., 2004). Thus, a clear picture of the various elements of synchronization and the underlying mechanisms involved has yet to emerge.

To summarize, several important physiological characteristics have come to light from in vivo analyses. These include clear ideas of the timing or rhythm of OXT neuronal bursts and their relationship to the stimulus input as well as their relationship to the MER. The concepts of summation, gating and synchronizing mechanisms have been well supported. It is clear from osmotic manipulations (Brimble and Dyball, 1977) that normal milk ejection bursts can arise out of slow-irregular as well as fast-continuous background firing rates. Microdialysis studies in the SON (Neumann et al., 1994) and PVN (Bealer and Crowley, 1998, 2001) also highlight the importance of numerous

neurochemicals in the MER, e.g. OXT, noradrenalin and histamine, which likely underlie the three mechanisms. What has not been obtained from in vivo analyses is any detailed information regarding the neural mechanisms that underlie the bursts themselves or the other phenomena just mentioned. Obviously, such information requires levels of analysis that are not routinely (if at all) attainable with in vivo extracellular recording methods.

Functional studies of milk ejection bursts and the MER

In vitro analyses

It seemed that brain slices would be the most reasonable in vitro model in which to investigate milk ejection burst-like firing patterns. In particular, slices containing the SON, taken from the brains of lactating rats, were deemed the most feasible place to begin these investigations. Some of the critical advantages of in vitro systems are control of the extracellular microenvironment, ability to record membrane and synaptic events and precise cellular identification. These allow for mechanistic analyses at cellular and molecular levels. The model chosen was acutely prepared slices from adult animals, which undergo little, if any, structural reorganization during the experimental period, i.e. they remain brain-like. Accordingly, they also appear to retain the functional characteristics of the animal from which they were dissected. Although during suckling many local neurochemicals are mobilized around OXT neurons, a common feature of their actions is reducing extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). It is known that the excitability of OXT neurons gradually increases between consecutive bursts during suckling (Wang et al., 1996b; Brown and Moos, 1997; Brown et al., 2000). This increased excitability may be due to intense activity-dependent reductions in $[Ca^{2+}]_o$, as documented in other systems (Parker, 2000). Increased excitability of SON (Li and Hatton, 1996) and other neuron types (Su et al., 2001) in vitro has been shown to accompany reductions in $[Ca^{2+}]_o$. Noradrenalin, acting on α_1-adrenoceptors, is known to be important in both parturition (Douglas et al., 2001) and lactation (Parker and Crowley, 1993), so an α_1-agonist was included in our manipulations. These two neurochemical factors in the SONs of lactating rats were singled out as being crucial to preparing OXT neurons for the production of bursts. Thus, by mimicking certain features of the microenvironment known to exist in the intact brains of lactating rats, OXT neurons were shown to emit milk-ejection-like bursts in vitro (Wang and Hatton, 2004).

Presented in Fig. 3 are data comparing many features of OXT neuronal bursts recorded in vivo and in vitro. Many of the burst characteristics are similar in the two models, a notable exception being the duration of the postburst inhibitory period. That this period is quite different in the two models suggests that it is normally under the influence of connections that were severed in the coronal slice preparation. Burst amplitudes also differed, but this was to be expected, since burst amplitudes are positively correlated with the number of suckling pups. Such stimuli arise from regions far outside of the hypothalamus and, thus, the brain slice preparation. That the bursts in vitro arose suddenly and intermittently, as do those in vivo, suggests that burst rhythm and gating mechanisms are either intrinsic to OXT neurons or reside within the local circuits contained in the slice. Some of the electrophysiological characteristics of OXT neuronal bursts, not observable from extracellular recordings, are shown in Fig. 4A. As they are in vivo, the OXT neuronal bursts recorded in vitro are distinct from the phasic bursts of AVP neurons. Three essential, distinguishing differences in spontaneously occurring bursts are that OXT neuronal bursts (1) are shorter in duration than AVP neuronal bursts, (2) have higher peak firing rates than AVP neuronal bursts and (3) are not superimposed on plateau potentials. It was clear from these findings that the milk-ejection-like burst was amenable to analyses at the membrane and synaptic level.

If milk-ejection-like bursts represent a pattern of activity dictated by the neurochemical microenvironment and local neural circuits, then they should not be restricted to the OXT neurons of lactating animals. OXT neurons in slices from male rats treated the same as those of the lactating animals might then display such burst patterns. A study designed to make this set of comparisons was done

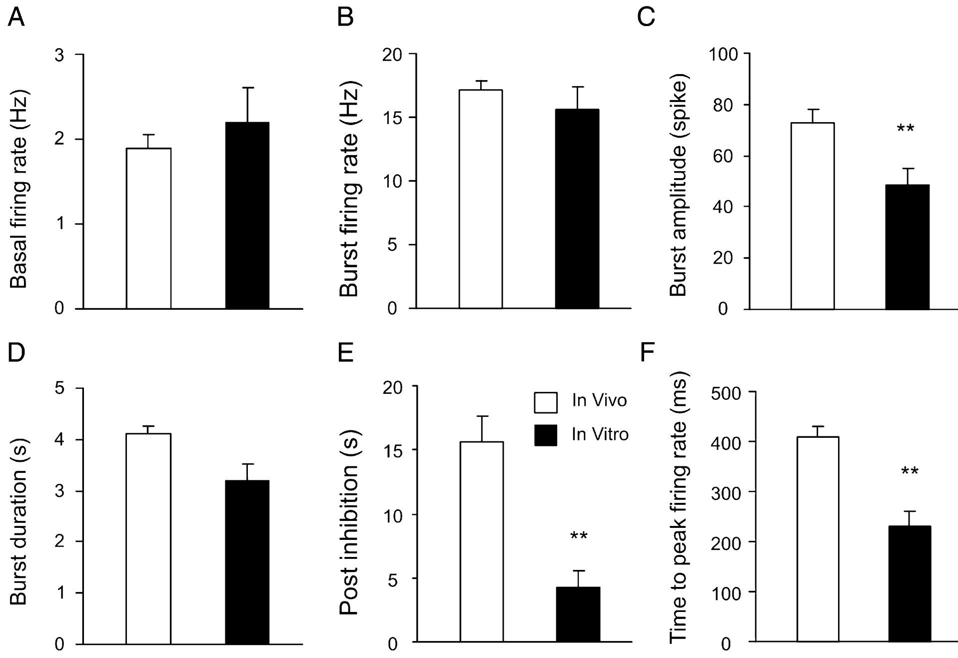

Fig. 3. Comparisons of several characteristics of bursts recorded from OXT neurons in vivo (open bars, $n = 26$) and in vitro (filled bars, $n = 19$). All neurons were from the brains of lactating rats. Adapted with permission from Wang and Hatton (2004).

(Wang and Hatton, 2005), and the findings affirmed that these burst patterns could be evoked in OXT neurons from the SONs of male rats (Fig. 4B). Although there were some differences in membrane phenomena between the bursts in male and lactating female brains, no differences were observed in burst characteristics that are most important in determining the amount of peptide release. For instance, the number of spikes in a burst, firing frequency within bursts and peak firing rates were similar in slices from males and lactating females. Such findings suggest that the mechanisms controlling OXT neuronal burst firing are independent of sex and reproductive state. They also suggest that gating mechanisms work at the level of OXT neurons, if the appropriate neurochemical environment is provided either by suckling or by artificial simulation.

Extracellular concentrations of OXT in the maternal SON increase significantly during a bout of suckling (Moos et al., 1989; Neumann et al., 1994), facilitating the activation of OXT neurons (Wakerley et al., 1994). This action can be blocked by intracerebroventricular injection of OXTR antagonists (Freund-Mercier and Richard, 1984; Moos et al., 1989). Excitatory effects of OXT on OXT neurons appear to stem chiefly from two sources: the downstream effects of activating OXTRs on the OXT neuron itself (Yamashita et al., 1987; Carter, et al., 2003) and a reduction in the amplitude of inhibitory postsynaptic currents (IPSCs) (Brussaard et al., 1996). Paradoxically, a reduction in excitatory drive was suggested by evidence showing that OXT reduced the amplitude of evoked excitatory postsynaptic currents (EPSCs) in SON neurons (Kombian et al., 1997). Perhaps OXT has excitatory or inhibitory actions, depending on immediately preceding events, i.e. the action may be time-and/or concentration-dependent. This might be inferred from the gradual OXT concentration increase during suckling, but previous studies, which had only used brief applications of OXT, would not have uncovered these possible dependencies. We, therefore, examined the effects of gradually increasing OXT concentrations on the excitability of OXT neurons in slices containing the SON, taken from lactating rats (Wang et al., 2006). First, our preliminary

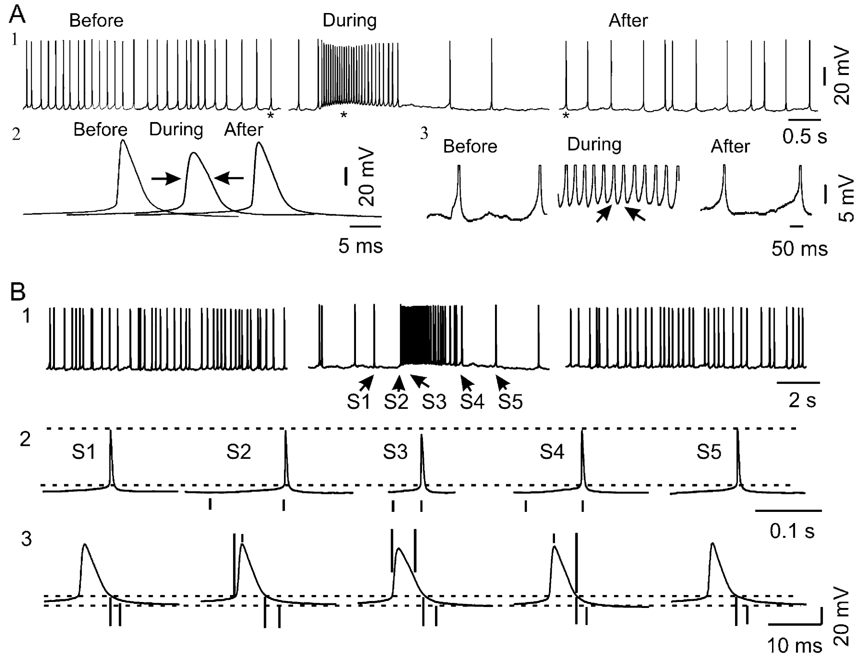

Fig. 4. Features of action potentials around and within bursts of OXT neurons. (A) Changes in membrane electrical events around a burst from the SON of a lactating rat. A1: Membrane events at 0–4 s before, during and 0–4 s after the burst, respectively. A2: Expansions of the spike(s) * in A1. Note the increased spike duration (between arrows) during burst. A3: Reduced width of AHP (between arrows) of spikes during burst in A1 (*). (B) Firing activities before, during and after a burst from the SON of a male rat. B1–2: S1–S5 indicate the spikes just preceding the burst (S1), the first spike (S2), the one at peak rate firing (S3), the spike at the end of the burst (S4) and the spike immediately following ~2-s postburst inhibitory period (S5). These are expanded in B2 and B3 to reveal the changes in spike and AHP characteristics. Data in (A) is adapted with permission from Wang and Hatton (2004), and in (B) from Wang and Hatton (2005).

patch-clamp recording studies had revealed that OXT could excite OXT neurons at concentrations far below what had been reported as the physiological range. Our experimental conditions, involving continuous aCSF perifusion of the slice, thereby removing endogenously released OXT from the cells at the surface, at least, probably contributed importantly to the sensitivity (<1 pM) of the OXT neurons to OXT application. Progressive increases in OXT concentrations (sub-picomolar to nanomolar) meant to mimic what occurs during a suckling bout, induced excitation and subsequent spike frequency reduction in OXT neurons. Consistent excitation was observed at the lower concentrations, reaching peak levels in the picomolar range before spike frequency reduction occurred in most neurons. The buildup of OXT concentrations progressively promoted depolarization of the membrane potential, spike broadening, decreases in spike amplitude and increases in the rise time of spike after hyperpolarizations that were unrelated to firing rate. OXT applied intermittently, for 5 min each, at progressively increasing concentrations evoked dose-dependent excitation, but not spike frequency reduction. Application of 1-pM OXT for 40 min simulated the effects of progressively increasing OXT concentrations. Vasopressinergic neurons were also activated by OXT but did not show spike frequency reduction. Consistent with presynaptic loci of OXT action, ionotropic glutamate receptor antagonists reduced OXT effects on firing rate, whereas bicuculline ($GABA_A$ receptor antagonist) did not change the excitatory effects. These results suggest that the specific autoregulatory effects of OXT, and perhaps other neuropeptides as well, are time- and concentration-dependent.

Cellular mechanisms underlying OXT-induced excitation

Although there seems to be only one main type of OXTR, these receptors are differentially expressed in different tissues, e.g. endometrium, mammary glands and brain (Gimpl and Fahrenholz, 2001). Downstream consequences of activating even identical receptors in inexcitable and excitable cells, of course, are likely to be different, but may be mediated by similar intracellular events. OXTRs belong to the $G\alpha_{q/11}$ class of G-protein-coupled receptors (GPCRs), as do α_1-adrenoceptors, activation of which is also excitatory. Such receptors initiate intracellular signalling cascades that activate protein kinase C (PKC), release Ca^{2+} from intracellular stores and activate extracellular signal regulated protein kinase 1/2 (ERK1/2) and increase prostaglandin (PG) expression. In slices from lactating rats, we investigated the downstream effects of OXT applications on both OXT and AVP neurons of the SON (Wang and Hatton, 2006). OXT application (10 pM, 10 min) significantly increased firing rates of OXT and AVP neurons, both of which were also found by immunocytochemistry to express OXTRs. Indomethacin, an inhibitor of PG synthetases (i.e. cyclooxygenase), blocked these increases. An OXTR (but not a V_1 receptor) antagonist blocked the OXT effects (Fig. 5) without blocking the excitatory effect of PGE_2 (not shown). Tetanus toxin, which prevents synaptic vesicle release, blocked OXT effects on fast synaptic inputs and firing activity of SON neurons, but not OXT-evoked depolarization, suggesting involvement of both pre- and postsynaptic neurons. Indomethacin also blocked the excitatory effects of α_1-adrenoceptor agonists, but not those of PGE_2, a non-$G_{q/11}$ GPCR-activating agent in the SON. Western blot analyses showed that OXT significantly increased cyclooxygenase-2 but not actin expression. OXT promoted the formation of filamentous actin (F-actin) networks at membrane subcortical areas of both OXT and AVP neurons. Indomethacin blocked the enhancement of F-actin networks by OXT, but not by PGE_2. Although this pathway involving mediation of excitability by PG/F-actin has been revealed to be an important pathway, it may be only one of the many. Others involving PKC and Ca^{2+} release from intracellular stores are likely to be important in the excitatory actions of OXT.

The above-cited work has confirmed the efficacy of OXT in evoking bursts. The major signalling pathway via OXTRs was believed to be the $G_{q/11}$

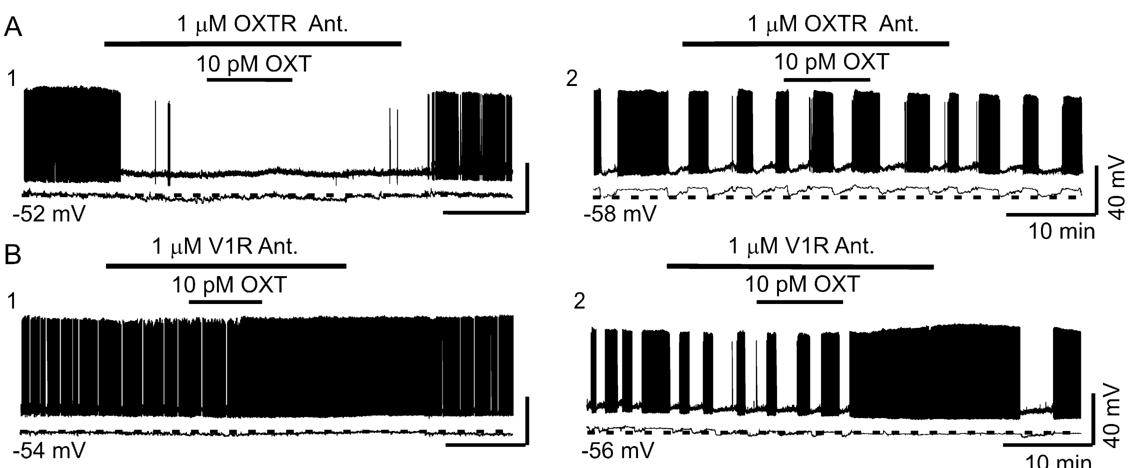

Fig. 5. Excitatory effects of OXT were mediated by OXT receptors (OXTRs). (A) and (B) show the effects of OXT on the electrical activity of SON neurons in the presence of OXTR antagonist (OXTR Ant.) and V_1 receptor antagonist (V_1R Ant.). A1 and A2 exemplify the effects of OXT on an OXT and an AVP neuron in the presence of the OXTR Ant., respectively. Note that neither OXT and AVP neurons showed excitatory responses to OXT. B1 and B2 exemplify the effects of OXT on an OXT and a AVP neuron in the presence of the V_1R Ant., respectively. Note that both OXT and AVP neurons were similarly excited by OXT. OXTR Ant., but not V_1 R Ant., blocked OXT effects. Adapted with permission from Wang and Hatton (2006).

type GPCRs and their downstream effectors (Gimpl and Fahrenholz, 2001). However, the potential role of $G\alpha_{q/11}$-associated $G\beta\gamma$ subunits in neurons remained to be examined. Theoretically, activation of GPCRs in OXT neurons may also release $G\beta\gamma$ subunits, as has been implicated in OXT actions on peripheral OXTRs (Hoare et al., 1999; Zhong et al., 2003). Contrary to classical views on GPCR signalling, the $G\beta\gamma$ subunits are the major mediators of OXT-evoked activation of ERK1/2 in myometrium (Zhong et al., 2003). G-protein subunits were also implicated in modulating electrical activities of neurons. In acutely dissociated hippocampal neurons, Ba^{2+} currents via N-type voltage-dependent Ca^{2+} channels were inhibited by activation of $G\beta\gamma$ subunits (Blumenstein et al., 2004). Amplitudes of the glycine-activated Cl^{-} currents were enhanced after application of purified G-protein $\beta\gamma$ subunits or after activation of a GPCR in the mammalian brainstem and spinal cord (Yevenes et al., 2003). Together, this evidence suggested that signalling pathways of $G\beta\gamma$ subunits might also be involved in burst generation. Therefore, we further explored burst firing and its

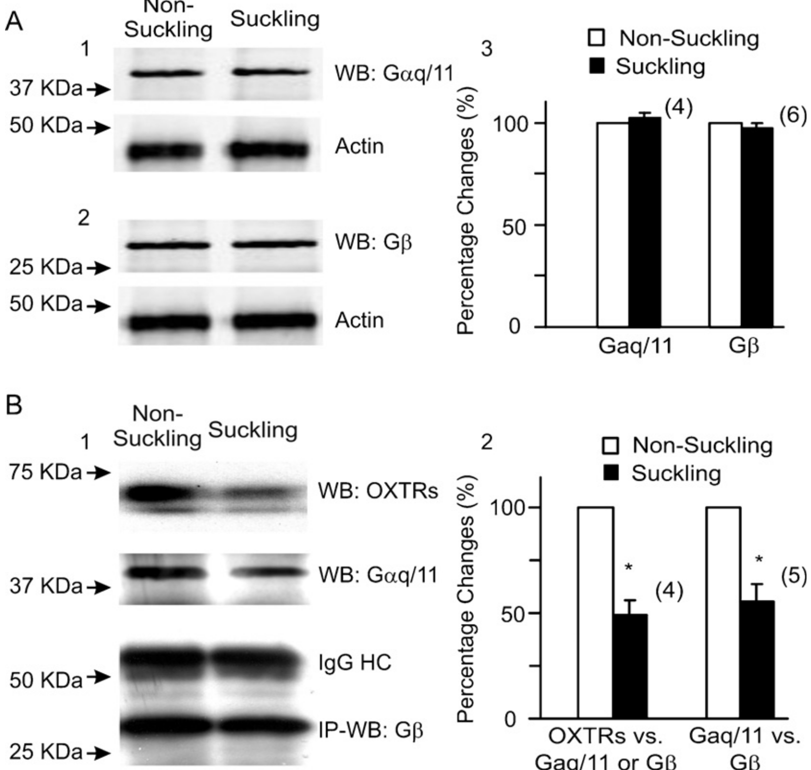

Fig. 6. Suckling mobilized OXTRs and $G_{q/11}$ proteins in lactating rats. (A) Western blot (WB) of $G\alpha_{q/11}$ and $G\beta$ proteins in non-suckling and suckling rats. A1 and A2 show examples of the effects of suckling on $G\alpha_{q/11}$ and $G\beta$ expression, in which actin was used as loading control (bottom bands). A3 is the summary graph of suckling effects on $G\alpha_{q/11}$ and $G\beta$ expression, respectively. The numbers in parentheses indicate pairs of observations. (B) Dissociation of $G\alpha_{q/11}$ subunit from OXTRs and $G\beta$ subunit. B_1. Immunoprecipitation (IP) of $G\beta$ subunit with rabbit antibody and WB detection of OXTRs (top panel) and $G\alpha_{q/11}$ protein (middle panel). IgG (lower panel, left column) and $G\beta$ protein (bottom) were used as negative control and loading controls, respectively. Non-suckling: After separation of mothers from 10 pups for 4 h; suckling: suckling of 10 pups for approximately 10 min within 1 min after occurrence of milk ejection reflex after a separation for 4 h. B2: Summary graph showing the relative changes in the molecular association between OXTRs with $G\alpha_{q/11}$ ($n = 3$) or $G\beta$ subunits ($n = 1$) and between $G\alpha_{q/11}$ and $G\beta$ subunits ($n = 3$ for $G\alpha_{q/11}$ IP, $n = 2$ for $G\beta$ IP). Since the changes were similar in the molecular association between OXTRs with $G\alpha_{q/11}$ or with $G\beta$ subunits, between IP of $G\alpha_{q/11}$ or $G\beta$ the data were pooled and paired t-tests performed ($^{*}p < 0.05$) after square root transformation of raw band densities. Adapted with permission from Wang and Hatton (2007).

underlying mechanisms in OXT neurons evoked by OXT at concentrations close to or within the physiological range in acute brain slices from adult lactating rats (Wang and Hatton, 2007).

Several important findings emerged. OXT was found to facilitate bursts by suppressing tonic glutamatergic EPSCs and enhancing high-frequency EPSC clustering, therefore being more apt to evoke bursts, which were eliminated when ionotropic glutamate transmission was blocked. Because OXT neurons and SON astrocytes are themselves probably glutamatergic, (Ponzio et al., 2006), the blockade of glutamatergic transmission and synaptic vesicle release may also include components from OXT neurons and astrocytes. We were also able to eliminate OXT-evoked bursts by intracellular blockade of the coupling between GPCRs and G-proteins with GDP-β-S. Although PKC is a potential link in this GPCR pathway, neither activating nor blocking PKC had effects on bursting. Emptying IP_3-sensitive internal Ca^{2+} stores with thapsigargin was also ineffective in eliminating OXT-evoked bursts. Conversely, application of the G-protein βγ-activating peptide, mSIRK, triggered bursts. That this was a postsynaptic effect was evident, as intracellularly loading an antibody against Gβ subunits inhibited burst generation. Presynaptic effects of mSIRK were also evident, as tonic EPSCs were suppressed and high-frequency clustering of EPSCs was facilitated. Western Blotting and co-immunoprecipitation were used to show that these same pathways operate in the milk ejection burst in vivo. A significant dissociation of OXTRs from $G\alpha_{q/11}$ subunits and from Gβγ subunits occurred during suckling, as compared to non-suckling lactating rats (Fig. 6).

These results indicated that $G\alpha_{q/11}$-associated Gβγ subunits play a dominant role in burst firing of OXT neurons at both pre- and postsynaptic sites. Further, we confirmed that suckling in intact animals mobilizes OXTRs and $G\alpha_{q/11}$-associated Gβγ subunits, which very likely function in the generation of milk ejection bursts in lactating animals.

Concluding remarks

Studies of the cellular and molecular mechanisms underlying the milk ejection bursts and reflex are still in their infancy, but progress has been made. Powerful experimental tools are now becoming available that should allow the rate of progress to accelerate, enabling us to explore areas that have hitherto been out of reach. Although this can be said for many areas of science, it seems clear that, for the neurohypophysial system, this is a particularly exciting time.

Abbreviations

AVP	vasopressin
$[Ca^{2+}]_o$	extracellular Ca^{2+} concentration
EPSC	excitatory postsynaptic current
ERK 1/2	extracellular signal-regulated protein kinases 1 and 2
F-actin	filamentous actin
GPCRs	G-protein-coupled receptors
IPSC	inhibitory postsynaptic current
MER	milk ejection reflex
mPVN	paraventricular hypothalamic nucleus, magnocellular division
NH	neurohypophysis
NS	neural stalk
OXT	oxytocin
OXTRs	oxytocin receptors
PGs	prostaglandins
PKC	protein kinase C
SON	supraoptic nucleus
VGL	ventral glial lamina

Acknowledgements

The authors' research is supported by National Institutes of Health grant NS009140. We thank P. Hatton for helpful comments on a previous draft of the manuscript.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 15

Oxytocin receptor signalling

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Abstract: The great diversity of the expression sites and proposed function of the oxytocin (OXT) receptor (OXTR) is paralleled by a diversity of its signalling pathways, many of which have still remained unexplored. We have used different approaches to discover novel pathways. By means of a phosphoproteomics approach, we have detected several distinct OXT-induced changes in tyrosine as well as threonine phosphorylation states of intracellular protein in myometrial cells. The most prominent change involved dephosphorylation of a 95-kDa phosphothreonine moiety. By N-terminal amino acid microsequence analysis, this moiety was shown to correspond to eukaryotic translation factor eEF2. This protein is a key regulator of protein synthesis and mediates, upon dephosphorylation, the translocation step of peptide chain elongation. These findings define a novel mechanism by which OXT assumes a so far unrecognized trophic function. We next elucidated the intracellular pathway(s) involved. We found that this effect is not mediated by any of the known pathways known to induce eEF2 dephosphorylation (mTOR, ERK1/2 or p38) but by protein kinase C. Consistent with this idea, we also found that direct stimulation of protein kinase C with a phorbol ester induced eEF2 dephosphorylation in myometrial cells. Using phosphoERK antibodies, we discovered by Western blotting that OXT induced phosphorylation of a higher molecular weight ERK-related protein. We were able to show that this band corresponded to "big MAP kinase1" or ERK5. ERK5 is part of a distinct MAPK cascade and promotes expression of the myosin light chain gene and plays an obligatory role in muscle cell development and differentiation. The role of ERK5 in myometrium has remained unexplored, but it is likely to represent an important novel pathway mediating OXT's effects on smooth muscle function. Further elucidation of these novel signalling pathways will have significant relevance for the development of novel pathway-specific OXTR agonists and antagonists.

Keywords: oxytocin; oxytocin receptor; trophic action; MAP kinases; ERK5; elongation factor 2; contraction in vitro assay

General thoughts on oxytocin receptor signalling

The oxytocin (OXT) receptor (OXTR) is a member of the family of G protein-coupled receptors (GPCRs) and, together with the three vasopressin receptor subtypes V1a, V1b and V2, the OXTR forms a subfamily of structurally

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DOI: 10.1016/S0079-6123(08)00415-9

related GPCRs. The OXTR mediates a very wide spectrum of physiological actions: OXTRs are expressed in different specific brain regions where they mediate behavioural functions, ranging from maternal behaviour to specific sexual and social behaviours. In the periphery, OXTRs mediate effects on uterine contractions, mammary gland milk ejection and differentiation, pituitary prolactin secretion, sodium excretion, T-cell function, cardiovascular control, cardiomyocyte and osteoblast differentiation and endothelial cell function (Zingg, 2000; Gimpl and Fahrenholz, 2001; Zingg and Laporte, 2003; Lim and Young, 2006). Finally, OXTRs are widely expressed in different cancers. Over 80% of breast carcinomas express the OXTRs, and OXTRs are present in endometrial adenocarcinomas, choriocarcinomas, glioblastomas, neuroblastomas and small cell lung carcinomas (Cassoni et al., 2004).

The OXTR has several features that render its study particularly interesting and relevant:

(1) The OXTR undergoes an exceptionally dramatic tissue-specific up- and down regulation. In the uterus, OXTR expression undergoes in all mammalian species studied a 10–100 fold upregulation during pregnancy (Soloff et al., 1979; Fuchs et al., 1984; Larcher et al., 1995). This striking regulation involves mechanisms at the level of gene transcription, mRNA stability and at the level of signal transduction and trafficking (Zingg and Laporte, 2003).

(2) The mixed OXT/vasopressin V1a receptor antagonist atosiban has proven to be clinically effective in inhibiting preterm myometrial contractions. In fact, atosiban is equally effective in delaying preterm birth as treatment by ritrodrine, an agonist specific for the β_2 adrenergic receptor (β_2AR). However, atosiban is accompanied with significantly less side effects (French/Australian Atosiban Investigators, 2001; The Worldwide Atosiban versus Beta-agonists Study, 2001). As a result, atosiban (Tractocile®) is currently approved in 29 countries in Europe for the treatment of preterm labour. A more specific OXT antagonist with a longer duration of action, barusiban, has also been developed (Reinheimer, 2007). On the other hand, OXT is the strongest uterotonic agent known and is used pharmacologically antepartum to induce or augment labour as well as postpartum to control postpartum hemorrhage (Saito et al., 2007). From a pharmacological point of view, the OXTR is the target of two opposing clinically relevant pharmacological strategies aimed at controlling uterine motility. From a physiological point of view, the efficiency of OXTR antagonists to block preterm labour contractions indicates that premature upregulation of the OXT/OXTR signalling system may be causally involved in provoking preterm labour contractions.

The great diversity of the expression sites and proposed function of the OXTR is paralleled by a diversity of its signalling pathways, many of which have still remained unexplored. A $G_{q/11}$-mediated pathway leading to stimulation of phospholipase C (PLC) inducing increased intracellular calcium and inositol trisphosphate production has been clearly defined (Zhong et al., 2003); however, the precise mechanisms by which OXT exerts its multiple biological actions have not been fully established.

In addition to activation of PLC, the OXTR is also able to activate the MAP kinases ERK1 and ERK2 in myometrial cells (Fig. 1A, B) (Ohmichi et al., 1995; Zhong et al., 2003). With respect to myometrial contractions, the OXTR and the β_2AR mediate opposite effects; however both receptors activate the ERK1/2 pathway. Yet, the dynamics of ERK1/2 activations differ between the two receptors. The activation mediated by the β_2AR is very transient and lasts <10 min, OXTR-mediated ERK1/2 activation is more prolonged and lasts >1 h (Fig. 1A, B). The MAP kinases ERK1/2 mediate several different biological actions. A main distinction exists between their nuclear and cytoplasmic actions. At the level of the cell nucleus, ERK1/2 mediate proliferative effects via mechanisms that include induction of c-fos synthesis (Karin, 1996). At the level of the cytoplasm, ERK1/2 effects have been proposed to mediate contractions and prostaglandin synthesis (Strakova

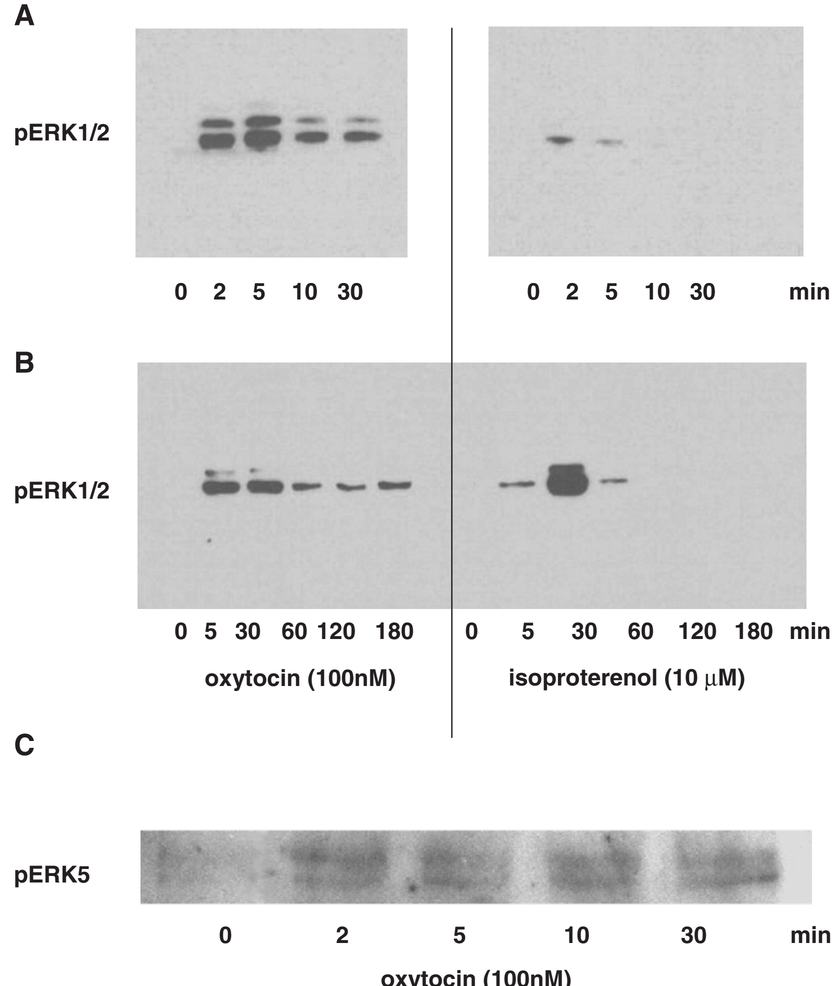

Fig. 1. (A, B) ERK1/2 activation in response to stimulation of OXTR (left) or the β_2 adrenergic receptor (right) in myometrial cells. Immortalized human myometrial hTERT-C3 cells (Devost and Zingg, 2007) were exposed to OXT or isoproterenol for the times indicated and phosphorylated ERK1/2 was immuno-detected by Western blotting using an anti-phosphoERK1/2 antibody (Cell Signalling Technology). (C) Kinetics of OXT-induced ERK5 induction in M11 myometrial cells. Cells were kept in serum-free medium for 48 h and OXT was added for the times indicated. Cell lysates were immunoblotted with an anti-phospho-ERK5 antibody (Cell Signalling Technology).

et al., 1998; Li et al., 2003). A mechanism has been proposed that accounts for nuclear versus cytoplasmic trafficking of ERK1/2 (Ahn et al., 2004). The model proposes two general pathways of ERK1/2 activation: (a) a very transient ERK activation (max ~ 5 min) that involves nuclear translocation of activated ERK leading to specific gene activation and a proliferative response; and (b) a sustained ERK activation (>30 min) that is associated with ERK retention in the cytoplasm leading to activation of cytoplasmic targets which, for myometrial cells, may include prostaglandin production and contraction. The model further suggests that the former pathway is G-protein dependent whereas the latter is β-arrestin mediated and G protein independent.

The prolonged kinetics of ERK1/2 activation by the OXTR suggests that OXTR-mediated ERK1/2 activation may include both nuclear and cytoplasmic effects, and that the sustained

activation of ERK1/2 is mediated via β-arrestin. Indeed, we have observed by confocal microscopy that the OXTR co-localizes with β-arrestin following internalization, suggesting that it internalizes into endosomes together with arrestin (unpublished data). On the other hand, the very transient ERK1/2 activation kinetics associated with the β_2AR suggest that the ERK1/2 action may be limited to nuclear effects. Studies determining to what extent OXTR-induced effects on ERK1/2 are β-arrestin mediated are currently in progress. Interestingly, Rimoldi et al. (2003) have recently shown that, if localized in caveolae (a type of lipid raft), the OXTR induces a transient ERK activation and transduces a mitogenic signal. However, if localized outside caveolae, the OXTR induced a sustained ERK response. Based on this finding, it appears that differential localization to membrane subdomains is an important determinant for OXTR function and has to be taken into consideration for a complete understanding of OXTR function.

Further exploration and characterization of different OXTR-dependent pathways is of major pharmacological importance. Recent advances in the field of drug action have made it clear that, for a given GPCRs coupled to different signalling pathways, a given receptor ligand may act as an antagonist for one pathway and as an agonist for another. Such a ligand is now referred to a "biased agonist" (Galandrin and Bouvier, 2006). It turns out that many ligands, initially classified as "antagonists" are in fact biased agonists, if a more comprehensive spectrum of signalling pathways is taken into consideration (Galandrin and Bouvier, 2006). As a case in point, the mixed OXTR/vasopressin V1a receptor antagonist atosiban mentioned above, has now been shown to be a biased agonist as well: Whereas it acts as an OXTR antagonist with respect to Gαq-mediated effects, it is a partial activator of the ERK1/2 pathway (Reversi et al., 2005).

Novel OXTR signalling target: eEF2

In an attempt to characterize more completely the spectrum of signalling pathways set into action following activation of the OXTR, we have determined, on a global level, to what extent OXTR signalling involves phosphorylation or dephosphorylation of specific signalling components. We analyzed OXT-induced changes in the protein phosphorylation pattern in lysates of CHO cells stably transfected with the rat OXTR (CHO-OTR cells), using one- and two-dimensional polyacrylamide gel electrophoresis in conjunction with specific phosphotyrosine and phosphothreonine antibodies. As a result, we have detected a number of distinct OXT-induced changes in the phosphorylation patterns of cellular proteins. These included pY100 and pY65, a 100 and a 65 kDa moiety, respectively, both phosphorylated on tyrosine. In addition, pT65, a 65 kDa protein phosphorylated on threonine, and pT95, a 95 kDa protein *de-phosphorylated* on threonine. Phosphorylation of pY100 and pY65 was very rapid and occurred within 2 min of OXT addition. The nature of these moieties is under investigation. Dephosphorylation of pT95 occurred between 5 and 60 min and was accompanied by the concomitant de novo phosphorylation of pT65 (Fig. 2). Maximum dephosphorylation of pT95 was observed at 20 min after OXT addition. Concomitantly, the

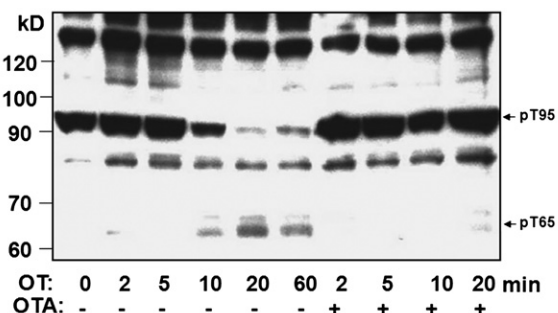

Fig. 2. OXT-induced changes in the overall threonine-phosphorylation pattern in CHO cells stably transfected with the rat OXTR (CHO-OTR cells). CHO-OTR cells were serum-starved for 24 h and treated for the times indicated with 100 nM OXT with or without prior treatment with the OXT-antagonist OTA at 1 μM. Proteins in cell lysates were separated by 7.5% SDS-PAGE and analyzed by immunoblotting using a specific anti-phospho-threonine antibody (Cell Signalling Technology). The position of the major de-phosphorylated substrate pT95 and the major phosphorylated substrate pT65 are indicated. Adapted with permission from Devost et al., 2005.

de novo phosphorylation of a band at M_r 65 kDa (termed pT65) was observed with a maximum of phosphorylation coincident with the maximal dephosphorylation of pT95. Additional bands were observed at 110 and 120 kDa that were rapidly phosphorylated within the first 2 min after OXT addition (Fig. 2).

We used ion exchange chromatography and one- and two-dimensional gel chromatography to purify pT95. By N-terminal microsequence analysis, the purified pT95 moiety was shown to correspond to eukaryotic translation factor eEF2. This protein is a key regulator of protein synthesis and mediates, upon dephosphorylation, the translocation step of peptide chain elongation (Browne and Proud, 2002). These findings define a novel mechanism by which OXT assumes a trophic function.

To confirm that pT95 corresponded to eEF2, we performed immunoblot analysis using a commercially available anti-phospho eEF2 antibody. If pT95 corresponded indeed to phospho-eEF2, then OXT should induce a decrease in phospho-eEF2 immunoreactivity that corresponded to the one observed for pT95. Moreover, we wished to determine whether this phenomenon was restricted to CHO-OTR cells or whether it could also be observed in untransformed myometrial cells. As shown in Fig. 3A, OXT induced a rapid decrease in phospho-eEF2 immunoreactivity in myometrial M11 cells. The time course of dephosphorylation corresponded to the one observed for pT95 in CHO-OTR cells. This finding provided further confirmation that the pT95 band corresponded to eEF2 and indicated that OXT-induced eEF2 dephosphorylation occurs in myometrial cells.

We next determined the dose–response relationship of OXT-induced eEF2 dephosphorylation. As shown in Fig. 3B, the maximum effective concentration of OXT was 10^{-8} M, and the efficiency of OXT induced dephosphorylation decreased with concentrations above 10^{-7} M. The fact that the same dephosphorylation was observed in the non-transformed myometrial cells as in OXTR-transfected CHO cells indicated that the observed OXT effect is physiologically relevant, since it can be mediated by the endogenous OXTR in a physiologically relevant cell type.

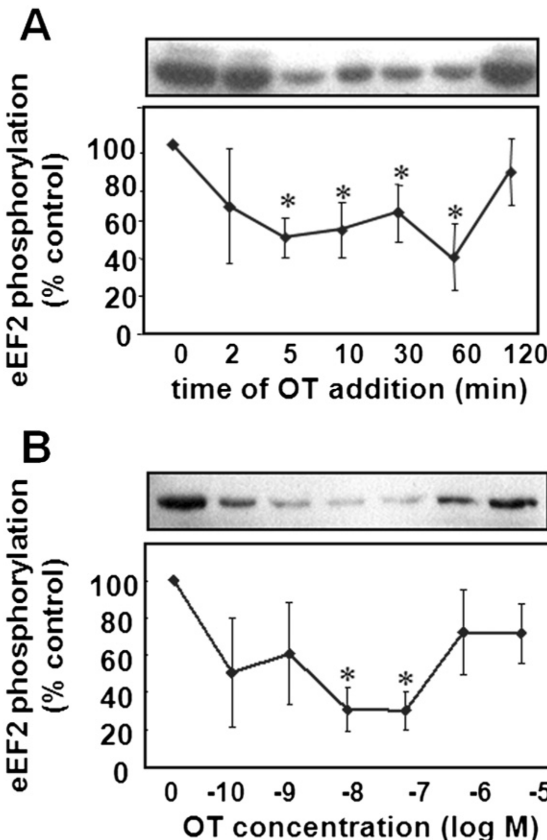

Fig. 3. OXT-induced eEF2 dephosphorylation in myometrial M11 cells. (A) time course of OXT-induced dephosphorylation. Cells were treated as in Fig. 1 with OXT for different times and eEF2 phosphorylation was assessed by immunoblotting using an anti-phospho-eEF2 antibody (Cell Signalling Technologies). Autoradiograms resulting from three independent experiments were analyzed by densitometric analysis using ImageQuant 5.1. The control values were set to 100%. Each point represents the mean ± s.e.m. A representative autoradiogram is shown in the top panel. (B) Dose/response curve of OXT-induced eEF2 dephosphorylation. eEF2 phosphorylation was assessed as in (A), and the means ± s.e.m. from three independent experiments were plotted against the OXT concentration used. A representative autoradiogram is shown in the top panel. Adapted with permission from Devost et al., 2005.

Because it is widely accepted that eEF2 dephosphorylation is accompanied by an increase in the rate of peptide chain elongation and, as a result, of protein synthesis, we wished next to determine to what extent OXTR activation was leading to a measurable increase in overall protein synthesis. To this end we determined the effect of 100 nM

OXT on the amount of [^{35}S]methionine incorporation into proteins in myometrial cells. OXT induced a significant 29% increase in the rate of total protein synthesis over a 2 h period (Devost et al., 2005). This stimulatory effect was similar to the one induced by insulin (32%). This finding indicated that the observed OXT-induced dephosphorylation of eEF2 is functionally meaningful and supports a novel role for OXT as a trophic agent in the myometrium.

In an attempt to delineate the pathway by which OXT exerts its effect on eEF2 dephosphorylation, we systematically explored each of the pathways know to stimulate eEF2 activity (Devost et al., 2008). This included (i) the serine/threonine protein kinase "mammalian target of rapamycin", mTOR, a pathway that is specifically inhibited by the bacterial natural product rapamycin (Browne and Proud, 2002); (ii) the MAP kinases ERK1 and ERK2, a pathway mediating the trophic effects of several GPCR agonists; (iii) "stress-activated protein kinase 4" (SAPK4 or p38MAPK).

First we analyzed the role of the mTOR pathway by using the selective mTOR inhibitor rapamycin. Whereas rapamycin pretreatment was effective in blocking insulin action on eEF2 dephosphorylation, it had no effect on the action of OXT on eEF2 dephosphorylation. To determine whether p38 activation was involved in eEF2 dephosphorylation, we used the selective p38 kinase inhibitor SB203580. However, blockage of this pathway also remained without effect on the ability of OXT to induce eEF2 dephosphorylation. We next blocked ERK1/2 activation with 1 μM of the specific MEK-1 inhibitor U0126. Although ERK1/2 activation was efficiently inhibited, application of this MEK-1 inhibitor, we did not observe any effect on the action of OXT on eEF2 dephosphorylation. Thus, none of the classically established pathways known to induce eEF2 dephosphorylation appeared to mediate the effect of OXT.

Due to its coupling to $G\alpha_{q/11}$, the OXTR is able to activate protein kinase C (PKC). Therefore we tested the hypothesis that the process might be mediated by PKC, although a link between PKC and eEF2 dephosphorylation has never been formally demonstrated. Indeed we found that application of the general PKC inhibitor Gö6983 completely blocked the effect of OXT on eEF2 dephosphorylation. To confirm further the hypothesis of a PKC-mediated effect of OXT on eEF2 dephosphorylation, we blocked PKC activation functionally by pretreatment with the peptide "myr-psi PKC", an *N*-myristoylated pseudosubstrate of PKC with specificity for the PKCα and PKCβ isotypes. Pretreatment with this peptide also abrogated the effect of OXT on eEF2 dephosphorylation. Consistent with a PKC-mediated effect, we found that direct phorbol ester-induced stimulation of PKC also led to a significant decrease in eEF2 phosphorylation (Devost et al., 2008).

As an indication for the physiological significance of these findings, we observed that in hTERT C3 cells, OXT led to a 38% increase in [^{35}S]-methionine incorporation into nascent proteins and that this effect was abrogated by preincubation of cells with the PKC inhibitor Gö6983, indicating that PKC activation represents an obligatory link connecting OXTR activation to increased protein synthesis via eEF2 dephosphorylation (Devost et al., 2008).

Novel OXTR-linked signalling pathway: ERK5

Using phosphoMAPK antibodies, we discovered by Western blotting that OXT induced phosphorylation of a higher molecular weight ERK-related protein. We were able to show that this band corresponded to "big MAP kinase1" or ERK5. ERK5 is part of a distinct MAPK cascade and promotes expression of the myosin light chain gene and plays an obligatory role in muscle cell development and differentiation. The role of ERK5 in myometrium has remained unexplored, but it is likely to represent an important novel pathway mediating OXT's effects on smooth muscle function.

We have confirmed that OXT induces ERK5 activity by using a specific phosphoERK5 antibody (Fig. 1C) as well as by an in vitro phosphorylation assay using the highly specific ERK5 substrate MEF2-C (unpublished). Our time course analysis using a phoshoERK5 antibody showed that OXT-induced ERK5 activation was

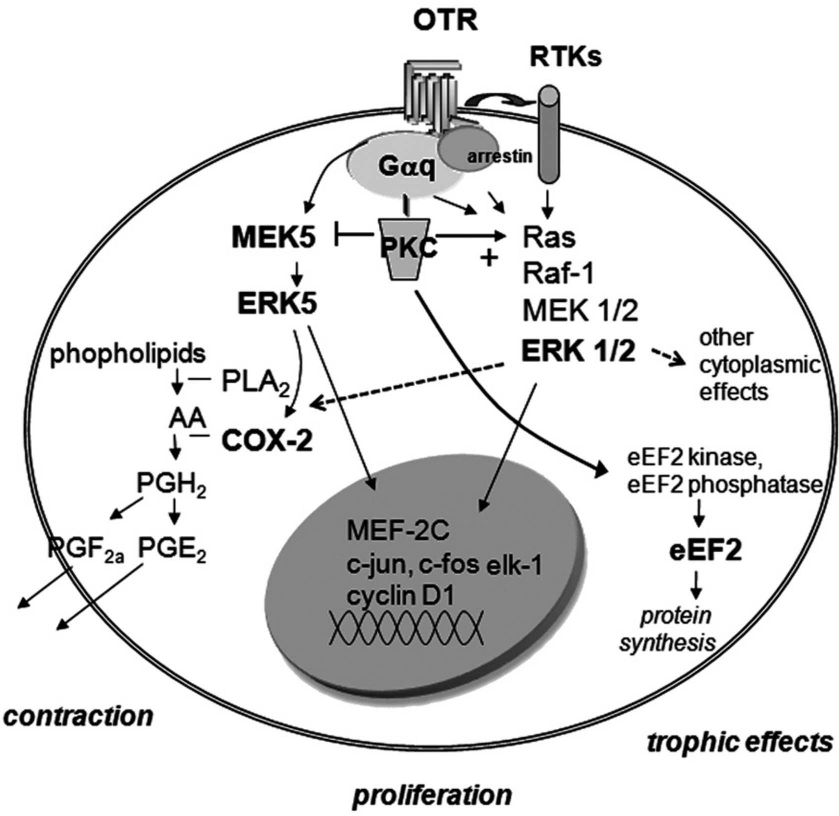

Fig. 4. Schematic diagram of novel and established OXTR-linked signalling pathways. This diagram illustrates some novel potential OXTR signalling pathways and their relationship to established pathways. Note also the bifurcation of ERK1/2 signalling into nuclear and cytoplasmic signalling. See text for further explanations.

sustained for at least 30 min (Fig. 1C). Interestingly, U0126, an inhibitor of MAPK kinases 1 and 2, blocked OXTR-mediated ERK1/2 activation, but at 10 μM, it also blocked ERK5 activation, implying that certain effects classically attributed to ERK1/2 may in fact be mediated by ERK5 (Mody et al., 2001). We also determined that the PKC inhibitor Gö6983, which blocked ERK1/2 activation, *increased* ERK5 activation (unpublished results). Thus, whereas PKC has been proposed to mediate OXT-induced ERK1/2 activity (Strakova et al., 1998), PKC has an *inhibitory* effect on OXT-induced ERK5 activation (Fig. 4). In this context, it is interesting to note that PKC activation has been reported to inhibit OXT-induced myometrial contractions (Phillippe, 1994). This finding is compatible with the idea that ERK5 rather than ERK1/2 activation could be involved in mediating OXT-induced contractions. In support of that idea, we found a dramatic increase in ERK5 phosphorylation in vivo in rat myometrium during pregnancy with a maximum at labour (unpublished). We hypothesize that the MEK5/ERK5 signalling cascade may be an important mediator of OXT actions, including prostaglandin synthesis, myometrial and myoepithelial cell proliferation and/or differentiation. If indeed ERK5 is involved in mediating specifically certain important uterine functions, the ERK5 signalling pathway could represent an attractive drug target.

In vitro contraction assay

To assess the involvement of ERK in mediating OXT-induced contractions, we have now developed

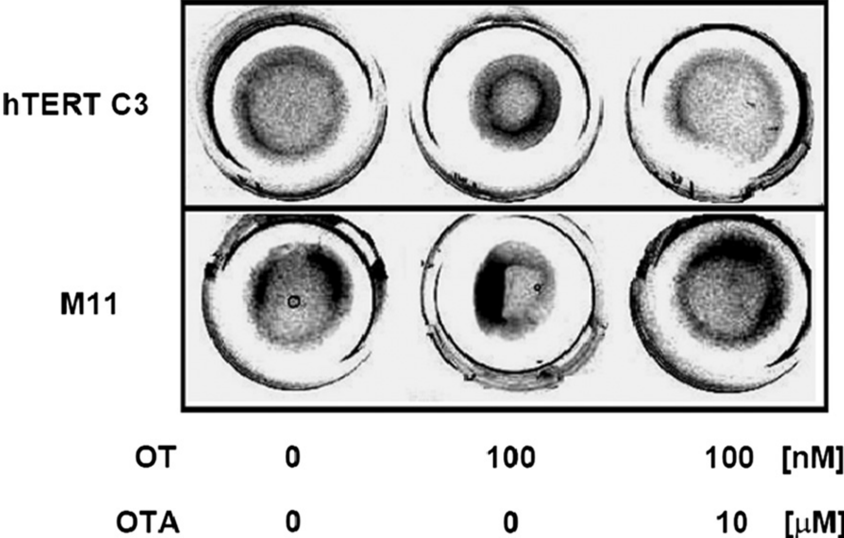

Fig. 5. OXT-induced contractions of myometrial cells in vitro. Collagen lattices were prepared and covered either by a layer of hTERT-C3 (top) or M11 (bottom) human myometrial cells. OXT alone or in combination with the OXT antagonist OTA was added as indicated. Lattices are visible in the center of each well. Adapted with permission from Devost and Zingg, 2007.

successfully a sensitive in vitro myometrial cell contraction assay (Devost and Zingg, 2007). In brief, myometrial hTERT-C3 cells were layered on top of collagen matrices in 24-well plates. Two hours after plating, the matrix was detached and allowed to float. OXT was then added at different concentrations for 1–18 h. Following fixation, collagen matrices were digitally scanned and the surface area was measured using the program ImageQuant 5.1. In the absence of OXT, cells induced a basal contraction of $48 \pm 3\%$. Addition of 100 nM OXT induced a significant increase in the contraction to $63 \pm 3\%$. Addition of the OXTR antagonist OTA in conjunction with OXT reduced the contraction to below the basal levels to $39 \pm 2\%$ (Fig. 5 and Devost and Zingg, 2007). These results indicate that we have developed a very sensitive and reproducible in vitro bio-assay to assess quantitatively the biological action of OXT on myometrial cell contraction. Since this assay is based on the use of myometrial cell lines, it offers not only full control of the extracellular environment but allows also the possibility of genetically manipulating the cells used in the assay system. Adaptation of the assay for high throughput analysis is currently in progress.

Conclusions

Further elucidation of novel signalling pathways linked to the OXTR will have significant relevance for the development for novel pathway-specific OXTR agonists and antagonists. The effect of new drug candidates will have to be assessed with respect to each of these additional pathways. This is particularly relevant in the light of the recent realization that receptor ligands can act in a pathway-specific fashion and exert differential agonistic or antagonistic effects on distinct pathways linked to the same receptor.

Abbreviations

β_2AR	beta-2 adrenergic receptor
eEF2	eukaryotic elongation factor 2
ERK 1/2	extracellular signal-regulated kinases 1 and 2

ERK 5	extracellular signal-regulated kinase 5
GPCR	G protein-coupled receptor
MAPK	mitogen-activated protein kinase
MEF2-C	myogenic enhancer factor 2-C
mTOR	mammalian target of rapamycin
OXT	oxytocin
OXTR	oxytocin receptor
PKC	protein kinase C
PLC	phospholipase C
s.e.m.	standard error of mean

Acknowledgement

Supported by a grant from the Canadian Institutes for Health Research (CIHR).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 16

Neurosteroids are excitatory in supraoptic neurons but inhibitory in the peripheral nervous system: it is all about oxytocin and progesterone receptors

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Abstract: Neuroactive steroids synthesized from the brain or peripheral sources are called neurosteroids. Beside their common nuclear effects, they are considered to be potent neuromodulators, acting rapidly mainly in a non-genomic manner, either through allosteric regulation of ionic channels, or through membrane-bound steroid receptors. In contrast to the situation in the adult, the neurotransmitter GABA is excitatory during development and plays a trophic role, in particular inducing calcium signals necessary for the regulation of excitability and neuronal maturation. We demonstrated that the primary metabolite of progesterone (Proges), allopregnanolone (Allo), evoked a robust Ca^{2+} influx in foetal hypothalamic neurons and in postnatal supraoptic nucleus (SON) neurons. In the latter, this led to oxytocin and arginine vasopressin release. Interestingly, these responses were $GABA_A$ and oxytocin-receptor-dependent. Allo is a well-known positive allosteric modulator of $GABA_A$ receptors. It is noteworthy that two other steroids, Proges and 17-beta-estradiol, displayed the same effect on Ca^{2+} and oxytocin release but to a lesser extent. Importantly, no effect was observed in adult neurons from the SON, or in neurohypophysial axon terminals, regardless of the stage. The molecular mechanisms of the neurosteroid actions are multifaceted and depend on the type of cells, and are thus extremely interesting and challenging. In the peripheral nervous system, Allo and Proges surprisingly inhibited the GABA-induced Ca^{2+} increases in embryonic dorsal root ganglion neurons. We propose that this rapid, reversible and dose-dependent phenomenon (at very low concentrations) was mediated by membrane Proges receptors, since transcripts for a newly discovered receptor protein, 25-Dx, were detected in our model. Recently, novel families of membrane steroid receptors, activating intracellular-signalling pathways such as MAP kinases, have been identified and described. This opens new perspectives to understand the intracellular machinery involved in the interaction between neuropeptides and neurosteroids, two major regulators of hypothalamo-neurohypophysial system development.

Keywords: $GABA_A$ receptor; allopregnanolone; progesterone; non-genomic effect; hypothalamic neurones; neurohypophysis; dorsal root ganglia; intracellular calcium; development

Introduction

The hypothalamo-neurohypophysial system is a major integrative centre of the brain, releasing

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DOI: 10.1016/S0079-6123(08)00416-0

pivotal neuropeptides and regulating essential life functions, such as parturition, lactation and antidiuresis. It represents an outstanding model in neurobiology because of its remarkable morphology, organization and capacity for plasticity. The understanding of its onset would shed more light on the mechanisms leading to dysfunctions and pathologies in the central nervous system (CNS).

Based on a plethora of data, it is generally accepted that neurosteroids represent a new class of neuromodulators that can rapidly alter neuron excitability via non-genomic mechanisms. However, although an increasing amount of data is currently available on the adult stage, the actions of steroid hormones during the development of the nervous system are multifaceted and still not well understood. We suggest that the rapid membrane effects (either excitatory or inhibitory) of neuroactive steroids support the molecular mechanisms involved in the excitability and activity of young neurons. The aim of this review is to emphasize the cross-talk between neuropeptides and neurosteroids in the developing nervous system, in particular by comparing CNS neurons from the hypothalamo-neurohypophysial system (supraoptic nucleus, SON and neurohypophysis) to the peripheral nervous system (PNS) neurons (dorsal root ganglion, DRG).

Neurosteroids: a new class of neuromodulators

The term ‘‘neurosteroids’’ was introduced by Baulieu (1981) to describe the steroids synthesized in brain tissue from cholesterol (Fig. 1) or from steroid hormone precursors (Guennoun et al., 1997; Keller et al., 2004; Belelli and Lambert, 2005). According to their effects they can be considered as ‘‘neuroactive steroids’’, a definition that includes all steroids that are active on neuronal tissue whether or not they are synthesized in the brain, by glial cells (Garcia-Segura et al., 1995; Baulieu, 1997) and by some neuronal populations (Sakamoto et al., 2001) or derived from peripheral sources.

Besides the classical genomic actions of steroids, these substances can rapidly alter the excitability of neurons. In fact, the ability of certain steroids and their metabolites to influence non-genomically a range of brain activities, including firing rate of neurons, induction of sedation, anaesthesia, neurosecretion and behavioural changes, is now well accepted (Schumacher, 1990; Paul and Purdy, 1992; Spindler, 1997; Wakerley and Richardson, 1998; McEwen and Alves, 1999; Toran-Allerand et al., 1999; Leng, 2000). Nevertheless, the molecular machinery underlying these phenomena remains controversial.

Non-genomic effects of progestins

The work of Ramirez et al. (1990) was one of the first to demonstrate that progesterone (Proges) and its metabolites can modify neuronal secretion by acting through a non-genomic transmembrane-signalling mechanism. They postulated that Proges could open Ca^{2+} channels of nerve terminals, leading to a rapid neuronal secretion. Evidence suggesting the role of Ca^{2+} in this phenomenon was provided earlier by Drouva et al. (1985), who showed that omission of Ca^{2+} in the medium, or addition of D-600, a Ca^{2+} channel blocker, antagonized the stimulatory effect of Proges on LHRH release from mediobasal hypothalamic slices (Fig. 1).

Allosteric modulation of ionic channels

A mechanism involved in the non-genomic effect of steroid, consists in the binding to a membrane-bound receptor complex, resulting in an allosteric modulation of major ligand-gated ion channels, such as GABA receptors in the adult stage (Belelli and Lambert, 2005) and/or NMDA receptors (Lambert et al., 1995; Rupprecht and Holsboer, 1999; Falkensten et al., 2000). Some steroids may either potentiate or inhibit NMDA receptor-mediated responses (Smith et al., 1987; Wu et al., 1991; Irwin et al., 1992, 1994; Park-Chung et al., 1994), and they can also have agonist or antagonist activity on $GABA_A$ receptors (Paul and Purdy, 1992). Interestingly, Oberwinkler et al. (2007) reported in a recent study the interaction of pregnenolone sulphate on TRPM3 channels in pancreatic β-cells. They found that pregnenolone sulphate reversibly activated the ion channel on a

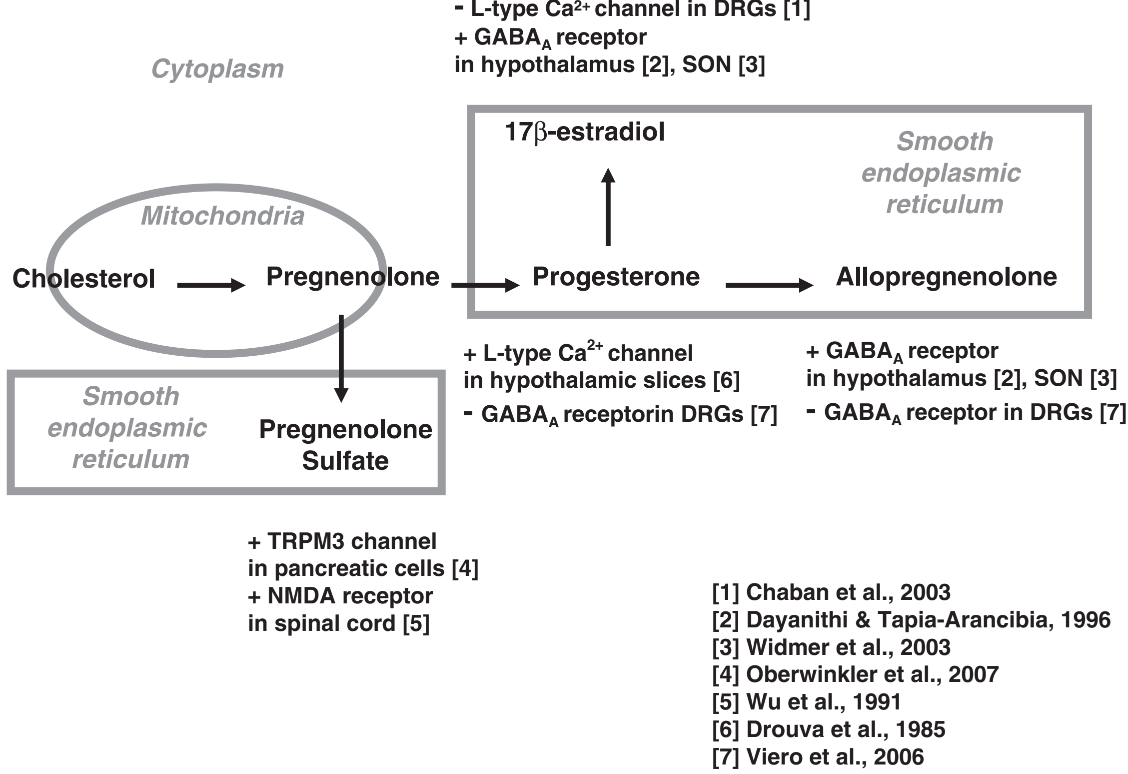

Fig. 1. Steroidogenesis and modulation of the excitability by steroids. This simplified scheme depicts the synthesis of steroids from cholesterol in the cell. In the mitochondria the side chain of cholesterol is cleaved by the enzyme P450scc. As a result, pregnenolone is produced and the further steps of steroidogenesis take place in the smooth endoplasmic reticulum via successive enzymatic reactions. The steroids synthesized by the neuron are then exported to act on other distant cells but can also work in a paracrine/autocrine manner. Steroids modulate differentially the activity of ionic channels in the nervous system as it is described in the figure (references can be found in the corner on the right hand side in the bottom part). Each arrow represents enzymatic pathways. The symbol "+" indicates "activation" of the channel/receptor and the symbol "–" indicates "inhibition" of the channel/receptor.

millisecond time scale (Fig. 1). Moreover, since these channels are highly permeable to Ca^{2+}, pregnenolone sulphate was shown to induce a rise of the intracellular Ca^{2+} concentration in cell lines derived from rat endocrine organs where transcripts for TRPM3 were detected and functional channels were identified.

Membrane receptors: Pgrmc1, Pgrmc2 and mPRs

The classical intracellular Proges receptor (iPR) isoforms iPR_A and iPR_B have been extensively studied (Schrader et al., 1972; Christensen et al., 1991), whereas membrane receptors mediating the rapid effects of Proges are still not well defined. Recent studies on rapid effects of steroids have been focused on two families of membrane receptors: Pgrmc1 and 2 (Meyer et al., 1996; Gerdes et al., 1998) and the progestin receptors mPR, members of the seventransmembrane-spanning domain receptors family (Zhu et al., 2003). Pgrmc1, the "Proges receptor membrane component 1", shows homology with the transmembrane domain of the precursor for the interleukin-6 receptor and a conserved consensus sequence found in the cytokine/growth factor/prolactin receptor superfamily (Selmin et al., 1996). In the rat, a Pgrmc1 homologue, protein 25-Dx, has two Proges-binding sites: one site of high affinity with an apparent K_d of 11 nM and one of low affinity with an apparent K_d of 286 nM (Meyer et al., 1996). Tissue expression of Pgrmc1 in mice during development is ubiquitous,

especially at the adult stage: it is expressed in adrenal cortex (Raza et al., 2001), liver (Nolte et al., 2000), hypothalamus (Krebs et al., 2000) and dorsal horn (Labombarda et al., 2003). It is noteworthy that transcripts of Pgrmc1 gene were also detected by Konno et al. from RIKEN in 2000 in spinal ganglia of 12 days old rats (unpublished results). Furthermore, Sakamoto et al. (2004) detected that Pgrmc1 was expressed in neonatal rat Purkinje cells precisely at the period when they synthesized Proges, and when they responded to this steroid. In contrast, little is known about Pgrmc2, though it derives from the same ancestral gene and shares similarities in topology (Cahill, 2007). Its functional characterization is still under investigation.

Recently, a unique family of membrane progestin receptors (mPRalpha, mPRbeta and mPRgamma) was identified, which may be responsible for mediating rapid, non-genomic actions of progestins in a variety of target tissues. In a study by Hanna et al. (2006), the mPRalpha and mPRbeta isoforms from zebrafish were rapidly and specifically activated by 4-pregnen-17,20beta-diol-3-one (17,20beta-DHP), and showed high affinity (mPRalpha, $K_d = 7$ nM; mPRbeta, $K_d = 12$ nM), saturable and displaceable binding of 17,20 beta-DHP to single-binding sites. The results of this study suggested that zebrafish mPRalpha and mPRbeta signal in a similar manner upon progestin binding, resulting in rapid activation of MAPK and downregulation of adenylyl cyclase activity, implying activation of a G_i protein, especially for the mPRalpha isoform.

While the direct binding of progestins to ionic channels and especially $GABA_A$ receptors leads to the potentiation of the response of $GABA_A$ receptors (Belelli and Lambert, 2005), membrane steroid receptors are good candidates for the mediation of other pharmacological action, including inhibitory effects.

$GABA_A$ and free cytosolic Ca^{2+}: a developmental pas-de-deux

It is now well established that in contrast to its inhibitory role in adult neurons, the neurotransmitter GABA is excitatory at the embryonic stage and therefore leads to the entry of Ca^{2+} into the cytosol of the cell. Intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ changes play a major role in the control of several neuronal functions (Burgoyne, 2007), notably during the development of the CNS (Ciccolini et al., 2003; Zamoner et al., 2007) and the PNS (Dayanithi et al., 2006) and also at some point in aging (Verkhratsky and Toescu, 1998; Toescu and Verkhratsky, 2003). The excitatory action of GABA could play a trophic role promoting synapse formation (Ben-Ari, 2002). It appears that GABA signalling is essential during neural development and proliferation, particularly when interacting with neurosteroids (Gago et al., 2004).

In the following sections, we highlight interesting insights arising from the comparison between the development of the CNS and the PNS, regarding the action of progestins on GABA and Ca^{2+}, two major components of neuronal maturation.

Development of the nervous system

Central nervous system: example of the hypothalamo-hypophysial complex

Wang et al. (1995) demonstrated that 17-beta-estradiol (17BE) could induce acute exocytosis of oxytocin (OXT) and arginine vasopressin (AVP) from the dendrites of adult hypothalamic neurons in female rats, but had no effect on release from neurohypophysial axon terminals. In foetal rat hypothalamic neurons, allopregnanolone (Allo), the primary metabolite of Proges, induces a rapid and large increase in $[Ca^{2+}]_i$ through activation of voltage-gated Ca^{2+} channels mediated by interaction with $GABA_A$ receptors (Dayanithi and Tapia-Arancibia, 1996).

In another study (Widmer et al., 2003), we demonstrated that in SON isolated from 3–4 weeks or less old rats, all three neurosteroids (Allo, Proges, 17BE) induced OXT release, but only Allo induced significant release of AVP. Surprisingly, in these very young rats, Allo-induced OXT release was inhibited by $GABA_A$ receptor antagonists as well as by an oxytocin receptor (OXTR) antagonist (Fig. 6A, B). In contrast, in SON from adult rats Allo-induced OXT release was much smaller, and was enhanced in the presence of bicuculline. The

$GABA_A$ receptor agonist muscimol (Musci) also induced OXT release from SON in young rats, but had no effect in adult rats. OXT cells isolated from young rats showed an increase in $[Ca^{2+}]_i$ in response to both Allo and Proges (Fig. 3A), but also to Musci. Allo had no effect on $[Ca^{2+}]_i$ or on the release of OXT or AVP from neurohypophysial axon terminals in either young or old rats. We conclude that, in very young rats, (i) neurosteroids induce OXT release from the SON by a mechanism that partly depends on the presence of GABA, which in young rats is depolarizing to OXT cells, and which also partly depends upon endogenous OXT, and (ii) the effect of Allo on OXT release changes with age, as the functional activity of $GABA_A$ receptors changes from excitation to inhibition of OXT cells.

We showed that, in young rats, large amounts of OXT can be released from the somato-dendritic compartments of SON neurons — much more than in adult rats (Widmer et al., 2003). This release can be evoked by a variety of neurosteroids, but particularly potently by Allo. The ability of Allo to evoke OXT release from the SON was dose-dependent. The effect was produced by concentrations as low as 10 nM, suggesting that this effect might be physiologically relevant.

In part, the actions of Allo in young rats are direct actions on the OXT cells; Allo evoked an increase in $[Ca^{2+}]_i$ in OXT cells from 14-day-old rats that was partly independent of external Ca^{2+} entry, suggesting a contribution from intracellular Ca^{2+} stores, but this response also involves mechanisms that depend on Ca^{2+} entry through voltage-gated channels in SON neurons. However, the data showed that OXT release from the SON in young rats depends largely on the presence of GABA and OXTR (Fig. 7A). Interestingly, activation of OXTR leads to the mobilization of intracellular Ca^{2+} from inositol triphosphate ($InsP_3$) stores, which primes the releasable pool of OXT in the dendrites and thus makes dendritic OXT available for release in response to subsequent spike activity (Ludwig et al., 2002).

It seems possible that the Cl^- gradient in these cells may be relatively late to mature, in which case GABA may be functionally excitatory in these cells well into postnatal life. Certainly this seems the simplest explanation for a stimulatory effect of Musci in young rats that is lost in fully adult rats, and for a stimulatory effect of Allo that diminishes with age. In our hands, GABA appeared to be depolarizing to OXT cells in young rats since the $GABA_A$ agonist Musci evoked OXT release from the SON of young (but not old) rats, and evoked an increase in $[Ca^{2+}]_i$ in OXT cells isolated from young rats, but not in cells isolated from old rats. In the fully adult rat, $GABA_A$ receptor antagonists potentiated Allo-induced OXT release from the SON, consistent with a reversal of the effect of GABA with ageing.

The ability of Musci to stimulate OXT release from the SON in young rats, therefore, may reflect a depolarizing effect of Musci in young rats, suggesting a delayed maturation of the Cl^- transporter mechanisms in these cells (Widmer et al., 2003).

Peripheral nervous system: example of the DRG neurons

DRGs mediate nociceptive, mechanical and proprioceptive sensory modalities (Fig. 2). PNS structures are able to synthesize steroids: related activities have been reported in glial cells (Robert et al., 2001; Patte-Mensah et al., 2003). In addition, sensory neurons from embryonic DRG also express 3-hydroxysteroid dehydrogenase which converts pregnenolone to Proges (Guennoun et al., 1997). Proges has neurotrophic roles, multiple effects on glial cells and promotes myelination (Koenig et al., 1995; Baulieu et al., 1996) via its conventional iPRs (Chan et al., 2000). DRGs are one of the rare models where GABA remains a long-time excitatory agent even at the postnatal and adult stages (Stein and Nicoll, 2003).

Ca^{2+} plays a major role, especially in DRG neurons where Ca^{2+} channels seem to undergo reprogramming in terms of sensitivity and density over time (Kostyuk et al., 1993), and during cell-death processes (Verkhratsky and Toescu, 2003).

We presented evidence that neurosteroids affect the $GABA_A$-induced $[Ca^{2+}]_i$ transients in a rapid and non-genomic manner (Viero et al., 2006). In contrast to what has been observed in most neural tissues (in spinal chord neurons (Twyman and

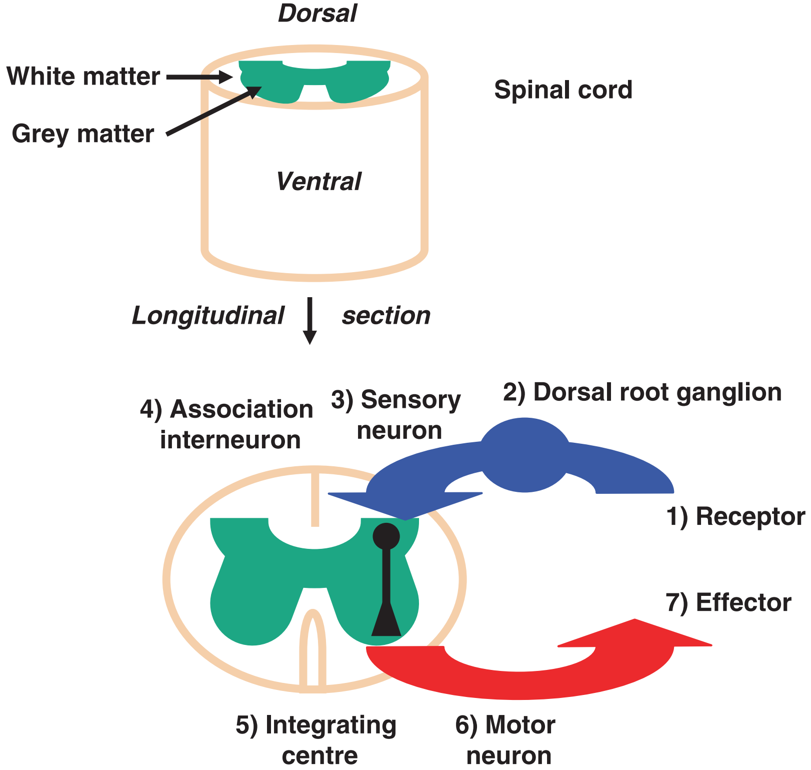

Fig. 2. Schematic representation of the integrated function of the somatosensory network. DRGs contain the cell bodies of primary afferent somatosensory and viscerosensory neurons (2), which receive nociceptive, mechanical and proprioceptive inputs (1) from periphery in order to forward them (3) to the brain via the spinal cord, but they are also involved in "reflex arch" phenomena (4, 5, 6, 7). (See Color Plate 16.2 in color plate section.)

Macdonald, 1992) and elsewhere (Belelli and Lambert, 2005)), treatment with Proges and its primary metabolite Allo inhibited the neuronal response to $GABA_A$ (Fig. 3B). To our knowledge, it is the first time that such an antagonistic effect was described for Proges and Allo on the $GABA_A$ receptor.

Furthermore, we demonstrated that $GABA_A$ response was to a large extent composed of Ca^{2+} influx through L-type Ca^{2+} channels induced by Na^+ channel-mediated depolarization (Viero et al., 2006). Intriguingly, in some neurons we could observe Ca^{2+} oscillations evoked by the application of Musci, the $GABA_A$ agonist (Fig. 4A). It is most likely that the initial Musci-induced Ca^{2+} transient triggered the phenomenon of Ca^{2+}-induced Ca^{2+} release (CICR) and thus activated in turn in these cells intracellular Ca^{2+} from $InsP_3$ stores, responsible for this typical oscillating release (Berridge, 2007). In addition, these oscillations were completely blocked in the presence of Proges (Fig. 4B), probably due to the initial inhibition of Ca^{2+} influx by the steroid, hence impairing the subsequent phenomenon of CICR. Though $InsP_3$ stores may not represent the major source of Ca^{2+} for embryonic DRG neurons, they seem to be present at early stages of development and participate as functional pools in adult DRG neurons (Svichar et al., 1997). More interestingly, it was shown 20 years ago that AVP and OXT caused an increase in the accumulation of inositol phosphates in DRG tissue of the rat (Horn and Lightman, 1987), probably acting through their respective receptors. Concerning the effects of AVP, these receptors had characteristics associated with the V1 subtype (Horn and Lightman, 1987). These

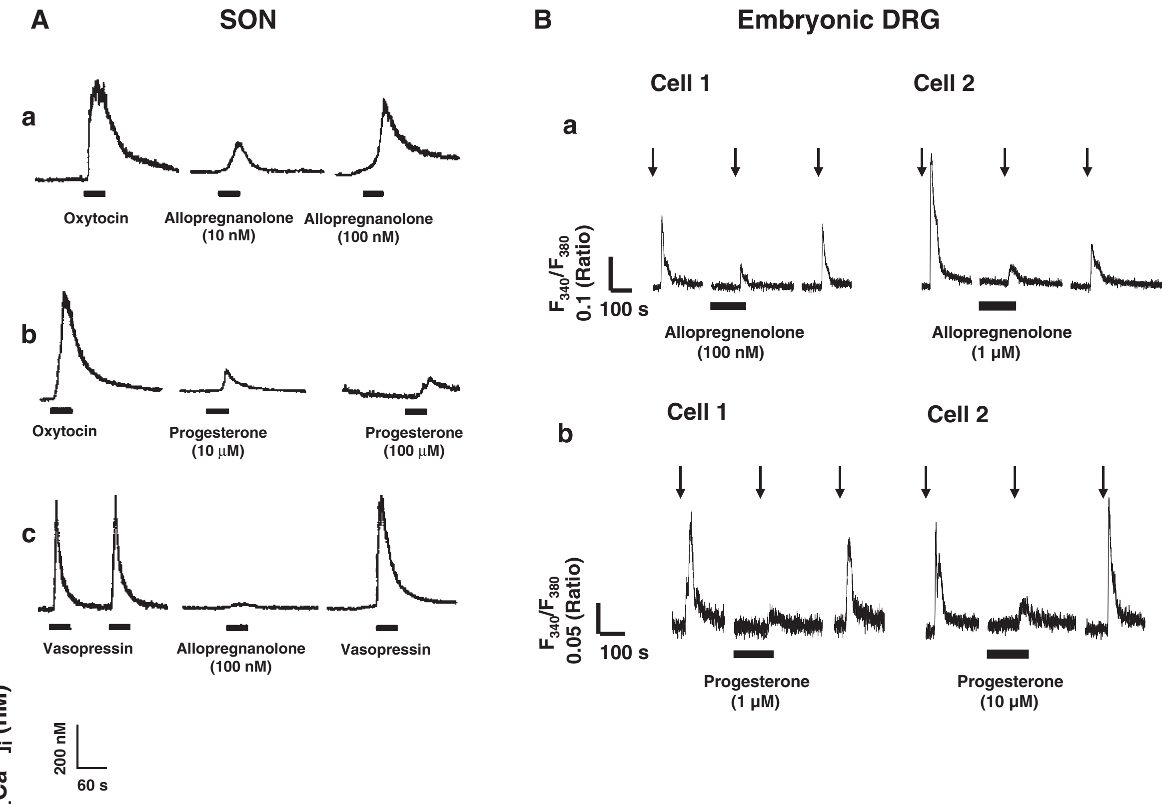

Fig. 3. Dual action of progestins on Ca^{2+} signals in the developing nervous system. (A) Effects of neuropeptides and progestins on $[Ca^{2+}]_i$ rise in isolated SON neurons. Young (a and b) and adult (c) SON cells displayed changes in $[Ca^{2+}]_i$ when they were challenged with (a and c) Allo (0.01 or 0.1 μM), (b) Proges (10 or 100 μM), (a and b) OXT and (c) AVP (0.1 μM). This panel was adapted with permission from Widmer et al. (2003). (B) Inhibitory effects of progestins on Musci-induced $[Ca^{2+}]_i$ increase. Representative traces of the fluorescence ratio F_{340}/F_{380} (arbitrary units) as a function of time. Embryonic E13 mouse DRG neurons were loaded with fura 2-AM and repetitively stimulated with Musci (vertical arrow: 10 μM) in the absence or presence of: (a) Allo (1 or 0.1 μM) or (b) Proges (1 or 10 μM). This panel was adapted with permission from Viero et al. (2006).

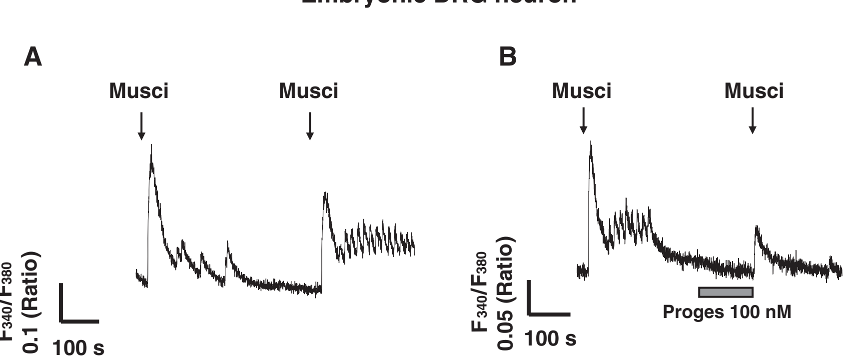

Fig. 4. Effects of Proges on Musci-induced $[Ca^{2+}]_i$ oscillations. Representative traces of the fluorescence ratio F_{340}/F_{380} (arbitrary units) as a function of time in an E13 mouse DRG neuron loaded with fura 2-AM (Viero et al., 2006). In this particular cell, Musci (10 μM) induced an oscillatory response. Proges (100 nM) resulted in a lower $[Ca^{2+}]_i$ increase after Musci stimulation and a loss of $[Ca^{2+}]_i$ oscillations.

findings corroborate our recent results obtained in adult DRG neurons. Small and medium diameter cell body adult DRG neurons (which usually transmit pain and thermal information; Scroggs and Fox, 1992) were challenged with either AVP or OXT (1 μM). Irrespective of the day of culturing (1–3 days) and the size of the cell body, both neuropeptides induced Ca^{2+} oscillations after more than 30 s of application and this phenomenon could be observed for more than 7 min (Fig. 5). The mean frequency of oscillations was 2.2 ± 0.4 Ca^{2+} transients per run of 60 s ($n = 11$ neurons). Taken together, it is highly conceivable that AVP and OXT mobilize $InsP_3$ stores even in embryonic DRG neurons (Fig. 7B), since these stores seem to be present at this early stage of development (Fig. 4) and since the neuropeptide receptors are already active in the young animal, especially in the SON (Fig. 6A, B). However, whether the corresponding neuropeptide receptors are expressed and responsive or not in embryonic DRG is still under investigation.

The new inhibitory effect of Proges on the $GABA_A$-induced Ca^{2+} response in embryonic DRG neurons was rapid and insensitive to RU38486. These properties excluded the possible involvement of the usual iPRs and led us to suggest the potential role of a membrane receptor. Among the putative Proges membrane receptors which could be involved in the rapid action of Proges, the screening of DRG transcriptomes performed during development (Mechaly et al., 2006) led us to focus on Pgrmc1, the orthologue of the rat 25-Dx protein. RT-PCR experiments at embryonic, postnatal and adult stages confirmed the expression of Pgrmc1 transcripts in DRG neurons in culture (Fig. 6C), therefore suggesting that the inhibitory effect of Proges on the $GABA_A$-induced $[Ca^{2+}]_i$ response could be mediated by these receptors. Interestingly and for the first time to our knowledge, transcripts for Pgrmc2 were also detected in this model (Fig. 6C). Nevertheless, these observations do not explain the lack of inhibition by Proges and Allo obtained in cultured adult DRG neurons (Viero et al., 2006), a regulation which could most likely take place at the protein level.

As Proges and Allo are present in the DRG environment at all stages from the embryo to the

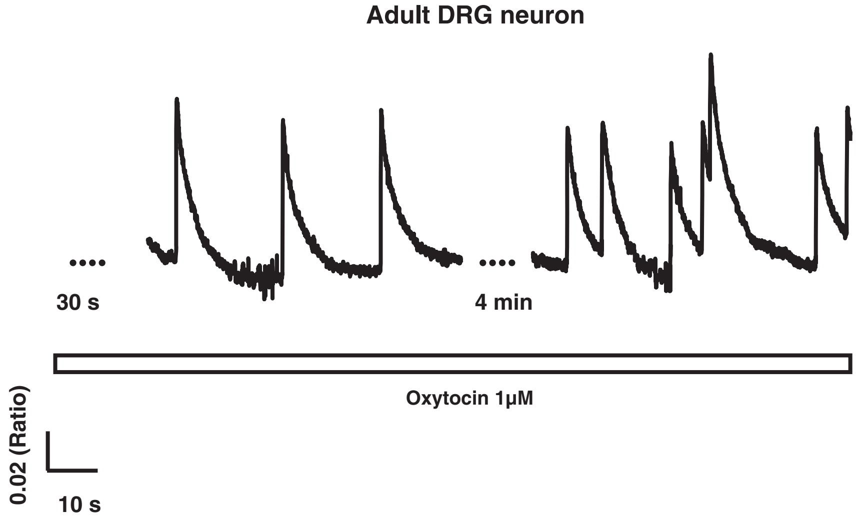

Fig. 5. OXT induces $[Ca^{2+}]_i$ oscillations in cultured adult mouse DRG neurons. Traces of the fluorescence ratio F_{355}/F_{380} (arbitrary units) as a function of time in an adult mouse DRG neuron (small-diameter cell body) cultured for 1 day and loaded with fura 2-AM (0.75 μM; Viero et al., 2008). In this particular cell, OXT (1 μM) induced an oscillatory response when applied during more than 6 min. Note that the cell was pre-incubated with OXT during 30 s prior to recording. A 4 min pause of recording was made between the two runs of the experiment while the neuron was still challenged with OXT. These data are representative of 11 DRG neurons responsive to neuropeptides (1 μM of OXT or AVP).

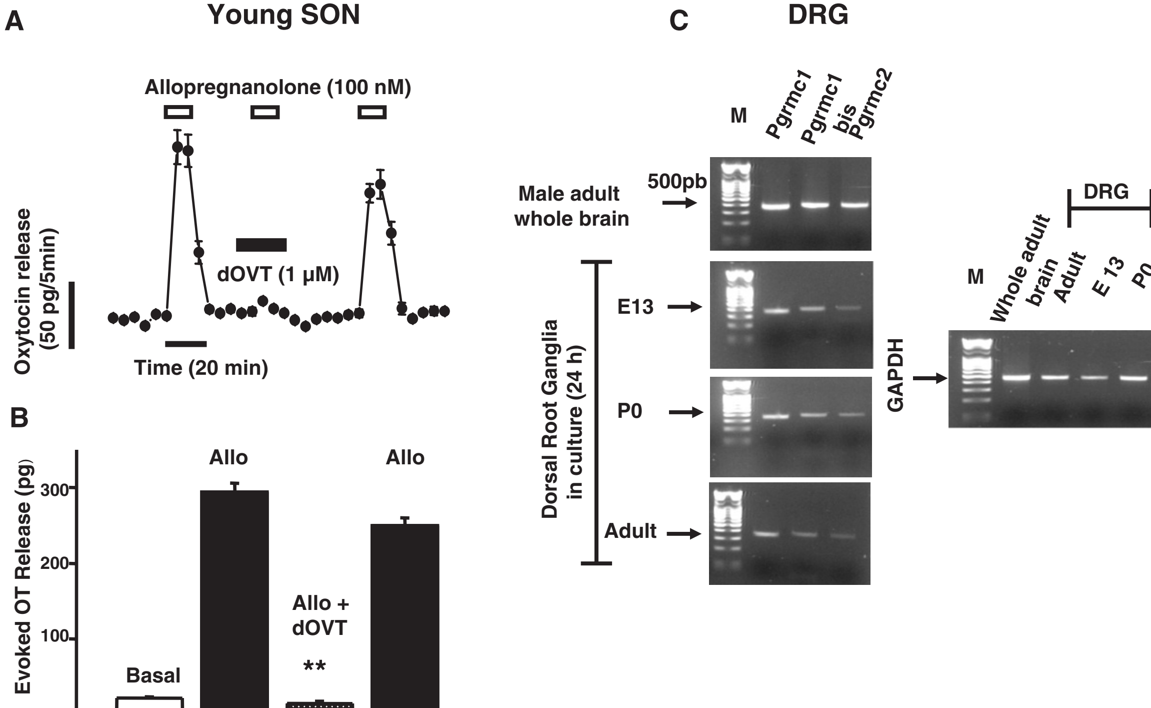

Fig. 6. Contribution of neuropeptide and progestin receptors in the modulation of neuronal excitability. (A) OXTR participates in the OXT release induced by Allo from the SON of young rats. The SON were incubated 10 min with an OXTR antagonist [d(CH_2)$_5$, Tyr(Me)^2, Orn^8]-vasotocin (dOVT, 1 μM) and then challenged with Allo. Data are the mean of four experiments (±SEM). This panel was adapted from Widmer et al., 2003 (courtesy of *J. Physiol.*). (B) Bar diagram illustrates the calculated evoked OXT release induced by Allo for the data obtained in A in the absence or presence of dOVT (significant inhibition: $p<0.001$). Note that after washing off the antagonist, dOVT, the response to Allo is nearly restored. Data are the mean of five experiments. (C) RT-PCR expression analysis of genes encoding for the Proges membrane receptors Pgrmc1 and Pgrmc2 in mouse DRG at different stages: embryonic (E13), postnatal (P0) and adult. RT-PCR product migration by electrophoresis on agarose (1 ×) gels containing ethidium bromide. Adult whole brain was used as positive control. GAPDH is the amplification-positive control. Molecular identity of amplicons was confirmed by sequencing (Genome express, France). M, size marker (MassRuler DNA ladder mix). Two sets of primers designed for the amplification of Pgrmc1 transcripts were used in this study: Pgrmc1 (AF042491): sense 5′AGCATTCGGTGGAGCATATC3′ and antisense 5′ATGGCTACACGCTGATCCTT3′; for Pgrmc1bis: sense 5′CCAAGACCTAGACACCTGAC3′ and antisense 5′GCTCTCCCACACACAGTAACT3′. Primers used for Pgrmc2 (BC044759) were: 5′ATGGGAAAGTCTTCGACGTG3′ and antisense 5′TCACACCCAAAGACTGGACA3′. Primers used for GAPDH (NM008084) were: sense 5′ACCACAGTCCATGCCAT-CAC3′ and antisense 5′TCCACCACCCTGTTGCTGTA3′. The expected lengths of resulting amplification are 400, 354, 383 and 453 bp, respectively. This panel was adapted with permission from Viero et al. (2006).

adult, the precise physiological role of $GABA_A$ inhibition by steroids remains to be elucidated. Nevertheless, according to recent insights in this field, we can speculate that progestins are likely to slow down the excitatory GABA action in order to counteract its maximal stimulation, which could otherwise probably lead to excitotoxicity. Therefore, we propose that these neurosteroids may have a neuroprotection action in the maturation of DRGs.

Strikingly, the screening of DRG transcriptomes performed by Mechaly et al. (2006) during development and after a peripheral nerve trauma revealed an induction of the expression of Pgrmc1 at the adult stage after axotomy (unpublished results), reinforcing the hypothesis that membrane-bound Proges receptors play a role in neural remodelling after injury.

Besides, it is noteworthy that potentiation by Proges (Schlichter and De Roo, 2004) and inhibition

by 17BE (Chaban et al., 2003) has been reported in the ATP-induced $[Ca^{2+}]_i$ increase in adult DRG neurons with reference to high-voltage-activated Ca^{2+} channels. All reported effects of progestins on the $GABA_A$ receptor indicate a potentiation of the response to GABA agonists (Majewska et al., 1986; Twyman and Macdonald, 1992; Park-Chung et al., 1994). It is generally assumed that the action of steroids involves direct binding to a specific site on the $GABA_A$ receptor. It seems reasonable that the inhibitory effect of progestins reported here does not involve binding to the same site, but rather implies an effect on Proges membrane receptors (Fig. 7B).

Physiological relevance of the models

Interestingly, two of the neuroactive steroids that are the most potent positive modulators of the inhibitory $GABA_A$ receptors, Allo (derived from Proges) and allotetrahydrodeoxycorticosterone (derived from deoxycorticosterone), have been detected in brain and plasma, where their levels fluctuated in response to stress and during the estrous cycles of female rats (Paul and Purdy, 1992). In normal male rats, for example, Allo is detectable in the cerebral cortex and hypothalamus at 2–4 nM, and its level being markedly and rapidly increased up to 10–20 nM following acute swim stress (Purdy et al., 1991) or up to 4–9 nM following acute electroshock (Korneyev et al., 1993).

Little information is available on Proges plasma concentrations in mice, but Proges levels vary in adult rats under basal conditions between 5 and 22 nM (Purdy et al., 1991) and increase with electroshock-induced stress to between 55 and 65 nM (Korneyev et al., 1993), with the water maze

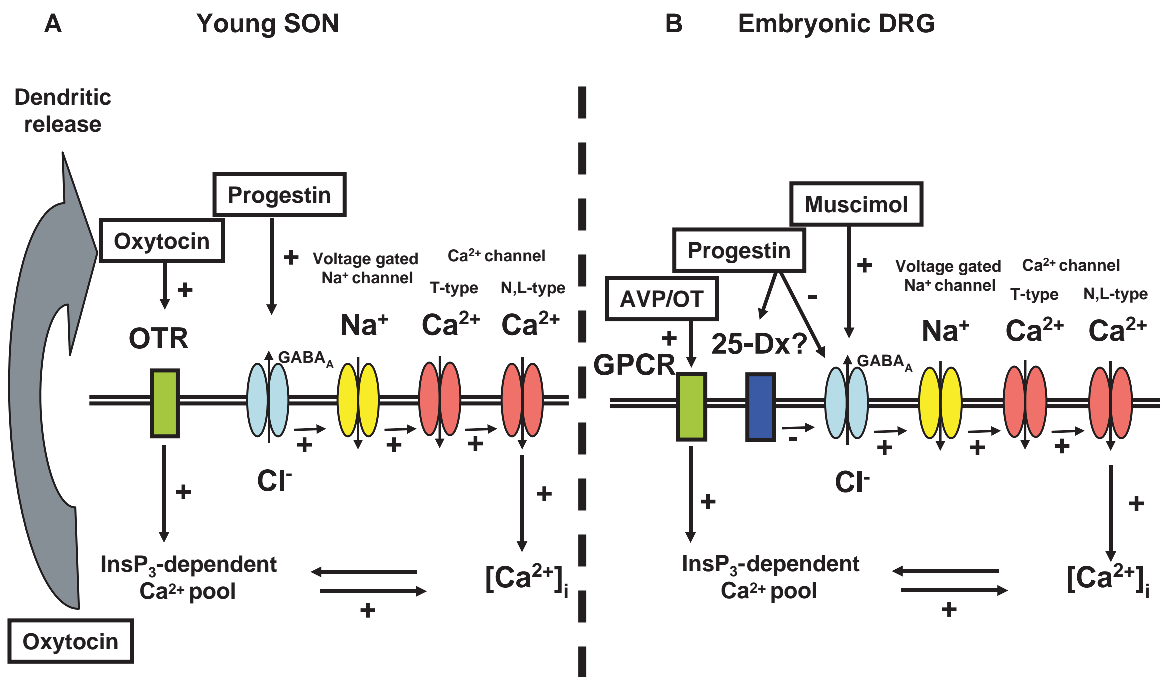

Fig. 7. Proposed mechanisms of progestin actions on central and peripheral developing neurons. (A) The figure illustrates the putative molecular machinery underlying the regulation of OXT release by progestins (Allo and Proges) in the SON of young rats. Dendritic release of OXT upon activation of $InsP_3$-dependent intracellular Ca^{2+} stores is regulated by both OXTR and depolarization-induced $[Ca^{2+}]_i$ elevation. (B) In embryonic DRG, progestins negatively regulate the excitatory effect of GABA. This unusual negative allosteric effect might be sustained by a new family of membrane Proges receptors (25-Dx) present in this system. In addition, we hypothesize that AVP and OXT mobilize $InsP_3$-dependent intracellular Ca^{2+} stores by acting through G_q protein-coupled receptors (GPCR) thus regulating $[Ca^{2+}]_i$ in DRG neurons, most likely already at early stages of development. (See Color Plate 16.7 in color plate section.)

test to between 20 and 42 nM (Purdy et al., 1991) and up to more than 1 μM during pro-estrus (Smith et al., 1975). Moreover, enzymes in the brain can generate active metabolites from the parent steroids (including Proges) and local concentrations of the neuroactive steroids may reach high nanomolar concentrations because of the contribution from the local production. We can therefore assume that we obtain effects with concentrations very close to the physiological range.

A crucial point needs to be addressed regarding the possible metabolism of Proges in our model. Indeed, a plethora of possible active steroids can be derived from Proges via well-known enzymatic pathways (Martini et al., 1993). Nevertheless, to date, no such rapid metabolism (120 s) has been reported in the literature, and especially not in DRG neurons. One of the first metabolites of Proges, isopregnanolone, has been tested, but it had no measurable effects on $[Ca^{2+}]_i$ responses (data not shown). In our study, Allo and Proges exert an inhibitory effect on the intracellular $[Ca^{2+}]_i$ response to $GABA_A$ receptor-agonists when applied at similar concentrations. This inhibition could either involve direct binding of the steroids to the $GABA_A$ receptor, with a negative instead of positive allosteric effect, or binding to an inhibitory membrane protein. In both cases, modulation of the responses to $GABA_A$-agonists by steroids could be linked to the unique subunit composition of the $GABA_A$ receptors found in embryonic DRG neurons (Laurie et al., 1992; Ma et al., 1993). This observation could also explain the lack of inhibition by Proges and Allo obtained in adult DRG neurons (cultured in the absence of neurotrophic factors), which suggests differences at the protein level.

Interactions between neurosteroids and GABA receptors have attracted particular attention in the case of magnocellular OXT neurons. OXT release from the dendrites of SON neurons acts back on the neurons to reduce the efficacy of GABA, and this effect is blocked by Allo, leading to the proposal that at term pregnancy, the fall in Proges precipitates enhanced excitability of OXT neurons through this effective GABA disinhibition (Brussaard et al., 1999, 2000). Thus, the actions of Allo on GABA effects are complex, and apparently involve protein kinase action (Fancsik et al., 2000).

Local OXT release plays a major role in the functionally labile organization of the SON in the adult female (Theodosis et al., 1986). In postnatal rats, OXT release has been suggested to play a role in the development of dendritic arborization (Chevaleyre et al., 2001, 2002). It, therefore, seems possible that, postnatally, OXT release in the hypothalamus evoked by GABA and by neurosteroids is analogously involved in the establishment of cellular architecture and functional synaptogenesis. Moreover, it has been shown recently that the combination of OXT and 17BE was essential for the fast development of functional GABA synapses in the adult SON (Theodosis et al., 2006). Another possible physiological role for OXT concerns the contribution of maternal OXT in the short-term switch from excitatory to inhibitory GABA synapses in foetal brain neurons during delivery, most likely in order to prevent excitotoxicity and injury during this phase (Tyzio et al., 2006).

Neuroactive steroid-signalling pathways

Apart from the classical genomic actions of Proges by its cytoplasmic receptor, it has been shown that, with a short delay of action, Proges is predominantly a synergistic allosteric effector of the $GABA_A$ receptor (Joels, 1997). In cultured hypothalamic neurons or in neurons freshly isolated from rat SON (Widmer et al., 2003), neurosteroids such as Proges, Allo and 17BE potentiate the $[Ca^{2+}]_i$ transients induced by a $GABA_A$-mediated depolarization (Dayanithi and Tapia-Arancibia, 1996). Besides GABA, non-genomic membrane-signalling mechanisms of Proges have also been demonstrated in the inhibition of Ca^{2+} release from the maintenance of water homeostasis after traumatic brain injury (Meffre et al., 2005). Two regulatory pathways have been proposed for the non-genomic action of Proges in the protection of ovarian granulosa cells from apoptosis and of hippocampal neurons in vitro from glutamate excitotoxicity. In the first study, Proges binding to a membrane receptor activates the mitogen-activated protein kinase cascade or MAPK (Peluso et al., 2003). The second proposed mechanism involves the extracellular signal-regulated kinase

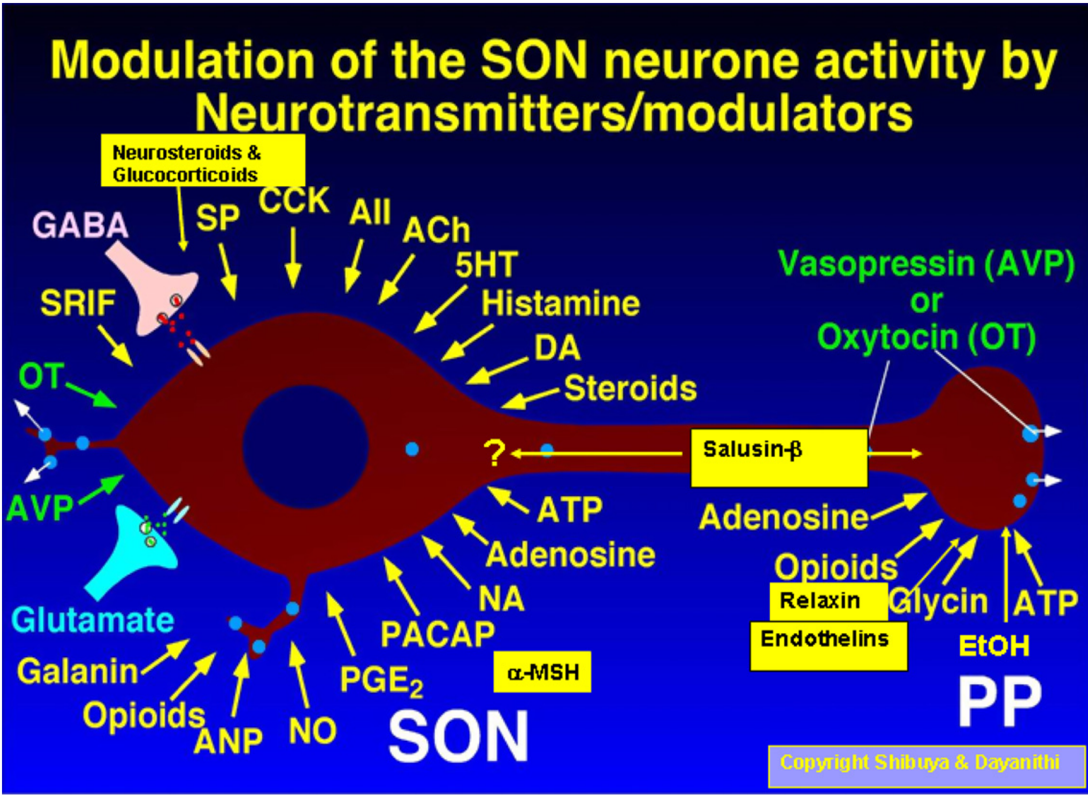

Fig. 8. Regulation of the SON neuron activity by neurotransmitters/modulators. The sketch summarizes the possible actors involved in the mechanisms controlling the excitability of neurons in the SON. It is of interest to note that many neurotransmitters/modulators (NT) are somehow involved in neuronal (SON) activity/regulation in some way or the other, only a few NT regulate the release of AVP/OXT at the level of neurohypophysis (PP). They induce different parallel signalling cascades that also display crosstalks, thus fine-tuning the neuronal function. This schematic diagram was originally made by Prof. Izumi Shibuya, Department of Veterinary Physiology, Tottori University, Japan and revised/modified accordingly to this review. (See Color Plate 16.8 in color plate section.)

(Erk) pathway and Bcl-2 expression with the Erk response occurring within 30 min (Nilsen and Brinton, 2002).

Another family of steroids, namely glucocorticoids, was shown to act rapidly on the SON by releasing endocannabinoids (anandamide and 2-arachidonoyl glycerol) from the soma and dendrites of magnocellular neurons, as retrograde messengers to modulate negatively the synaptic release of glutamate (Di et al., 2005). Remarkably, the same group had elucidated a mechanism in parvocellular neurons of hypothalamic paraventricular nucleus based on the interaction of glucocorticoids with a postsynaptic G-protein-coupled membrane receptor GR_{mb} that activates an intracellular pathway leading to the synthesis and the release of endocannabinoid (Di et al., 2003).

Hence, a plethora of modulators coexists within the SON and they all regulate in a concerted manner the excitability of the SON neurons at the somato-dendritic or axonic level (Fig. 8).

Conclusion

All these data strongly reinforce the idea of a putative dual effect of neurosteroids during the maturation of the nervous system. While progestins may play a role in neuroprotection during the development of the peripheral nervous system

(DRG), they exert an excitatory action in the brain of the young animal (SON). Despite a common mechanism of action leading to the regulation of GABA-induced Ca^{2+} release, it appears that different receptors underlie these phenomena in both cases, namely Proges membrane receptors in sensory neurons and OXT receptors in the hypothalamus.

Abbreviations

17BE	17-beta-estradiol
Allo	allopregnanolone
AVP	arginine–vasopressin
CICR	Ca^{2+}-induced Ca^{2+} release
CNS	central nervous system
dOVT	$[d(CH_2)_5$, $Tyr(Me)^2$, $Orn^8]$-vasotocin
DRG	dorsal root ganglion
GPCR	G_q protein-coupled receptors
$InsP_3$	inositol triphosphate
iPR	intracellular progesterone receptor
mPR	membrane progesterone receptor
Musci	muscimol
OXT	oxytocin
OXTR	oxytocin receptor
Pgrmc	progesterone receptor membrane component
PNS	peripheral nervous system
Proges	progesterone
SON	supraoptic nucleus

Acknowledgements

We thank Drs. Keith Langley (INSERM-U583, Montpellier, France) and Jeff Tasker, (Tulane University, New Orleans, USA) for their critical reading/pre-review of the manuscript. We are also grateful to Prof. Adolfo Cavalié (University Hospital, Homburg, Germany) who provided the adult mouse DRG neurons and to Dr. Ilana Méchaly (INSERM-U583, Montpellier, France) for her excellent expertize in molecular biology. We thank Mr. Will Veen (WillCo Wells B.V., Amsterdam, NL) for his generous gift of glass-bottom cover dishes for Ca^{2+} measurement experiments and a partial financial support to run this project. We wish to express our gratitude to all other authors of the work published in *The Journal of Physiology (J. Physiol. 2003; 548: 233–244; Publisher: Blackwell Publishing Ltd.)* and *Cell Calcium (Cell Calcium 2006; 40: 383-391; Publisher: Elsevier)*. We are also grateful to the Institute for Molecular Cell Biology (Homburg, Germany) which supplied the video imaging setup for Ca^{2+} measurements in Fig. 5. G. Dayanithi was partially supported by "Alexander von Humboldt Stiftung-Germany" to attend VIIth WCNH-Regensburg-2007 conference.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 17

Oxytocin receptors: ligand binding, signalling and cholesterol dependence

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Abstract: The G protein coupled oxytocin receptor (OTR) reveals some specific molecular and physiological characteristics. Ligand–receptor interaction has been analysed by photoaffinity labelling, site-directed mutagenesis, the construction of receptor chimeras and molecular modelling. Major results of these studies will be summarized. The N-terminus of the OTR is mainly involved in agonist binding. Notably, antagonists that are derived from the ground structure of oxytocin, bind the receptor at distinct sites partly non-overlapping with the agonist binding site. OTRs are able to couple to different G proteins, with a subsequent stimulation of phospholipase C-β isoforms. In dependence on G protein coupling, OTRs can transduce growth-inhibitory or proliferatory signals. Some evidence is provided that OTRs are also present in form of dimeric or oligomeric complexes at the cell surface. The affinity of the receptor for ligands is strongly dependent on the presence of divalent cations (Mg^{2+}) and cholesterol that both act like positive allosteric modulators. While the high-affinity state of the receptor for agonists requires divalent cations and cholesterol, the high-affinity state for antagonists is only dependent on a sufficient amount of cholesterol. Cholesterol affects ligand-binding affinity, receptor signalling and stability. Since the purification of the OTR has never been achieved, alternative methods to study the receptor in its native environment are necessary. Promising strategies for the site-specific labelling of the OTR will be presented. The employment of diverse reporter molecules introduced at different positions within the OTR might allow us in the near future to measure conformational changes of the receptor in its native lipid environment.

Keywords: oxytocin receptor; cholesterol; steroids; affinity state; bungarotoxin; Sfp phosphopantetheinyl transferase

Oxytocin

Oxytocin (OT) and oxytocin-like hormones are primarily produced in magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei and are secreted from the posterior pituitary into the systemic circulation in response to a variety of stimuli such as suckling, parturition or certain kinds of stresses. Maternal OT exerts a powerful action on foetal neurons by inducing a transient excitatory-to-inhibitory switch in their GABA signalling shortly before delivery. Reduced neuronal activity may protect foetal brain from hypoxic insults during delivery (Tyzio et al., 2006). OT is also synthesized in peripheral tissues, e.g. in uterus, testis and heart. Besides its classical

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DOI: 10.1016/S0079-6123(08)00417-2

functions, labour induction and lactation, OT plays an essential role in social behaviour, the oestrous cycle, penile erection and ejaculation (Gimpl and Fahrenholz, 2001). OT-deficient mice displayed impairments in milk ejection and social recognition, but surprisingly no defects in parturition (Nishimori et al., 1996). Mice deficient in the OT receptor (OTR) also exhibit normal parturition and defects in lactation and social behaviour (Takayanagi et al., 2005). Overall, the OTR system supports physiological processes associated with reproduction at several levels in all vertebrates.

All neurohypophysial hormones are nonapeptides with a disulphide bridge between Cys-1 and Cys-6 (Fig. 1). This results in a peptide constituted of a six-amino acid cyclic part and a C-terminal α-amidated three-residue tail. The peptides are classified into vasopressin and OT families, based on the amino acid 8. The vasopressin family contains a basic amino acid, whereas the OT family contains a neutral amino acid at this position. Isoleucin in position 3 is essential for stimulating the OTRs and Arg or Lys in position 8 for acting on vasopressin receptors. Thus, the difference in the polarity of these amino acid residues enable vasopressin and OT to interact with their corresponding receptors (Gimpl and Fahrenholz, 2001).

Oxytocin antagonists

Oxytocin antagonists are valuable substances for the treatment of preterm labour by reducing myometrial contractions. Previously, the antagonist $d(CH_2)_5[Tyr\text{-}(Me)^2,Thr^4,Orn^8,Tyr^9]$vasotocin has been widely used (Elands et al., 1988). However, it exhibits substantial vasopressin V1A antagonism (Manning et al., 1995). Atosiban, another peptide antagonist, was shown to reduce the contraction rate in preterm labour and is used clinically for the indication of delaying imminent preterm birth. However, atosiban also suffers from lack of specificity, e.g. it binds to the vasopressin V1A receptor (Akerlund et al., 1999) and its affinity to the OTR is only sevenfold higher compared with its affinity to the vasopressin V2 receptor. Recently, barusiban, a much more selective OTR antagonist, has been developed (Nilsson et al., 2003). Barusiban shows several times higher affinity and potency for the human OTR than vasopressin and any other OTR antagonist (Pierzynski et al., 2004). The structures of both atosiban and barusiban are depicted in Fig. 1.

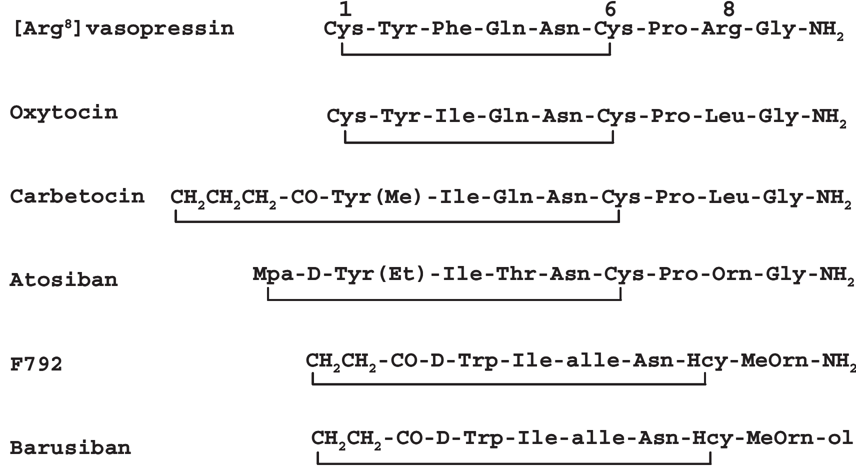

Fig. 1. Ligand of the oxytocin receptor. Arg8-vasopressin, agonist of the vasopressin receptors and partial agonist of the oxytocin receptor; oxytocin and carbetocin, agonists of the oxytocin receptor; F792 and barusiban, antagonists of the oxytocin receptor; F792-rhodamin, F792 antagonist with fluorophore rhodamine group; alle, alloisoleucine; Hcy, homocysteine; MeOrn-ol, N^{α}-methylornithinol; Mpa, 3-mercaptopropionic acid; Orn, ornithine.

Oxytocin receptors

The OTR belongs to the class A (rhodopsin-like) superfamily of G protein coupled receptors (GPCRs). Within this class the vasopressin/OTR forms a small family that comprises three types of vasopressin receptors, designated as V1A, V1B and V2, and the OTR. The vasopressin V2 receptor shows only 40% overall sequence identity with the OTR, but strongly discriminates between the ligands [Arg8]vasopressin and OT (structures in Fig. 1). [Arg8]vasopressin is bound with nearly 400-fold higher affinity than OT. The OTR binds OT and [Arg8]vasopressin with similar high affinity (Gorbulev et al., 1993; Akerlund et al., 1999). Thus, chimeric OT/vasopressin V2 receptor constructs can be used to identify ligand-binding domains. Employing displacement studies we have investigated to what extent the introduction of OTR domains into the vasopressin V2 receptor changes the affinity of various ligands structurally related to OT (Postina et al., 1996; Gimpl et al., 2005).

Ligand binding: agonists versus antagonists

Ligand–receptor interaction has been studied by photoaffinity labelling (Breton et al., 2001), site-directed mutagenesis (Fanelli et al., 1999; Hawtin et al., 2001), the construction of receptor chimeras (Postina et al., 1996; Gimpl et al., 2005) and molecular modelling (Fanelli et al., 1999; Hawtin et al., 2001). Major results of these studies are summarized in the receptor model shown in Fig. 2.

The binding profile for OT comprises the extracellular amino-terminus E1 and the extracellular loops E2 and E3 of the receptor. The contribution of the extracellular amino terminus for OT binding has also been detected by other studies (Hawtin et al., 2001; Wesley et al., 2002). In particular, the amino acid residue R34 within the E1 domain has been identified as essential for high-affinity OT binding using site-directed mutagenesis (Wesley et al., 2002). In all members of the neurohypophysial peptide hormone receptor family, an arginyl is conserved at this locus. This residue might be required for agonist binding throughout the receptor family. Interestingly, the binding profiles for OT and another agonist, carbetocin, are very similar (Gimpl et al., 2005). Carbetocin is a highly stable agonist with a long duration of action that binds to the OTR with 10-fold lower affinity as compared with OT (Engstrom et al., 1998). Overall, it appears that the N-terminus of the OTR is mainly involved in agonist binding and can not select between different agonists (Hawtin et al., 2001; Wesley et al., 2002). Glycosylation sites within the N-terminal part of the OTR are probably not involved in agonist binding. Receptor glycosylation may support efficient receptor trafficking and enhanced cell surface expression (Kimura et al., 1997; Wesley et al., 2002). As revealed by the crystal structure of rhodopsin, the N-terminus of GPCRs could form a compact domain which overlays the extracellular loops (Palczewski et al., 2000; Mirzadegan et al., 2003). According to molecular modelling, the OT-binding site is formed by the upper parts of transmembrane helices 3–7 and regions E2 and E3 (Fanelli et al., 1999).

Interestingly, OT antagonists have never been found to bind to the N-terminus of the OTR (Elands et al., 1988; Postina et al., 1996; Breton et al., 2001; Hawtin et al., 2001; Wesley et al., 2002; Gimpl et al., 2005). In studies with chimeric OT/vasopressin V2 receptor constructs, the competitive antagonist $d(CH_2)_5[Tyr(Me)^2,Thr^4,Orn^8,Tyr^9]$vasotocin (Elands et al., 1988) has been observed to bind at the transmembrane helices 1, 2, and upper parts of helix 7 (Fig. 2), separate from the agonist-binding domain (Postina et al., 1996). The photoreactive analogue of this antagonist interacted with a tripeptide motif in the upper part of transmembrane domain 3 of the OTR (Breton et al., 2001). The homologue tripeptide motif within the vasopressin V1A receptor has also been identified as a binding site for $d(CH_2)_5[Tyr(Me)^2,Thr^4,Orn^8,Tyr^9]$vasotocin (Breton et al., 2001). In terms of receptor specificity, results obtained with this antagonist are therefore difficult to interpret.

The binding domain of barusiban, the most specific OTR antagonist, clearly differs from the binding domain of the non-selective antagonist $d(CH_2)_5[Tyr\text{-}(Me)^2,Thr^4,Orn^8,Tyr^9]$vasotocin. For example, the tripeptide sequence ‘LVK’ at the

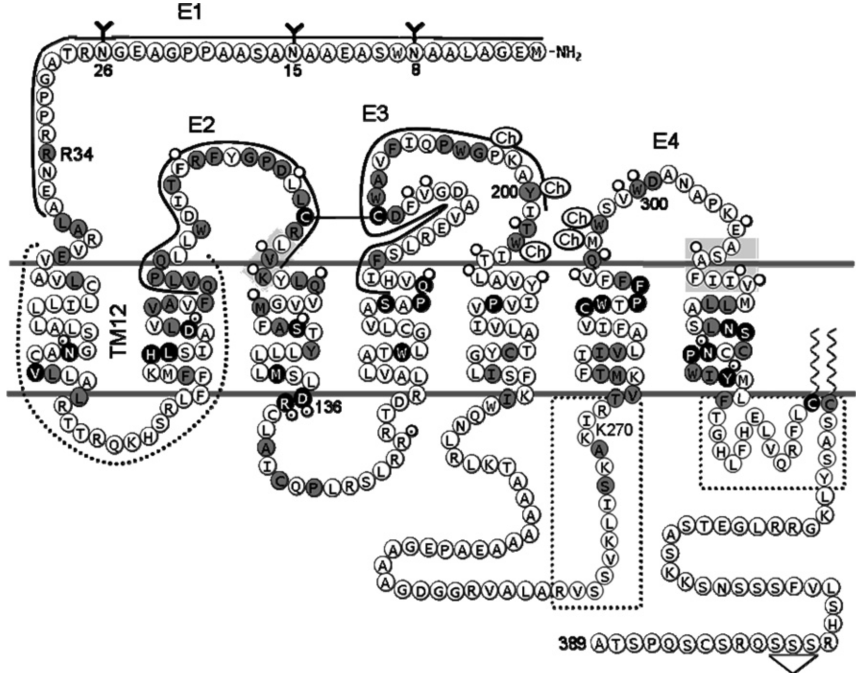

Fig. 2. The human oxytocin receptor and its putative binding domains for agonists, antagonists, G proteins and cholesterol. Black circles, residues conservative for the whole GPCR family; grey circles, residues conservative within the oxytocin/vasopressin receptor family; residues marked with 'Y', putative *N*-glycosylation sites; solid lines, oxytocin and carbetocin-binding domains, with extracellular domains E1, E2 and E3; dashed line, transmembrane helices 1 and 2 as part of barusiban binding; amino acids within grey boxes, previously defined binding sites for oxytocin receptor antagonists (Postina et al., 1996; Breton et al., 2001); open triangle, serine cluster putatively acting as retention signal of internalized receptor (Innamorati et al., 1998); residues marked with '○', oxytocin docking site according to molecular modelling (Fanelli et al., 1999); residues marked with '⊙', residues in the 'polar pocket site' according to molecular modelling (Fanelli et al., 1999); residues marked with 'Ch', cholesterol binding according to molecular modelling (Politowska et al., 2001); dotted rectangles, domains involved in coupling to $G_q\alpha$ (Hoare et al., 1999; Yang et al., 2002; Zhong et al., 2004; Zhong et al., 2007).

upper part of transmembrane domain 3 (Fig. 2) that has been identified as binding site for the non-selective antagonist by photoaffinity labelling (Breton et al., 2001), is probably not involved in barusiban binding because the transfer of the E2 domain into the vasopressin V2 receptor did not result in increased affinity for barusiban (Gimpl et al., 2005). Using the chimeric receptor approach, we provided evidence that the transmembrane domains 1 and 2 are in direct contact to barusiban (Gimpl et al., 2005). Possibly, hydrophobic parts of barusiban, e.g. D-Trp at position 2, interact with these transmembrane helices via hydrophobic interaction.

Overall, it is very likely that all ligands structurally related to OT/vasopressin can bind within a central receptor pocket of the OTR. Due to the anti-clockwise arrangement of the transmembrane helices of the OTR (Fig. 3), amino acid residues far apart within the primary sequence are spatially located close together. Different residues located at the central polar pocket site of the receptor can interact with OTR agonists or antagonists (Fig. 3). While agonists form contact sites with both the 'roof' (i.e. amino terminus) and the extracellular loop domains of the pocket, OT antagonists prefer contact sites at the bottom of the pocket. Barusiban might gain its increased affinity to the receptor via dipping into the bottom of the pocket between the transmembrane domains 1 and 2.

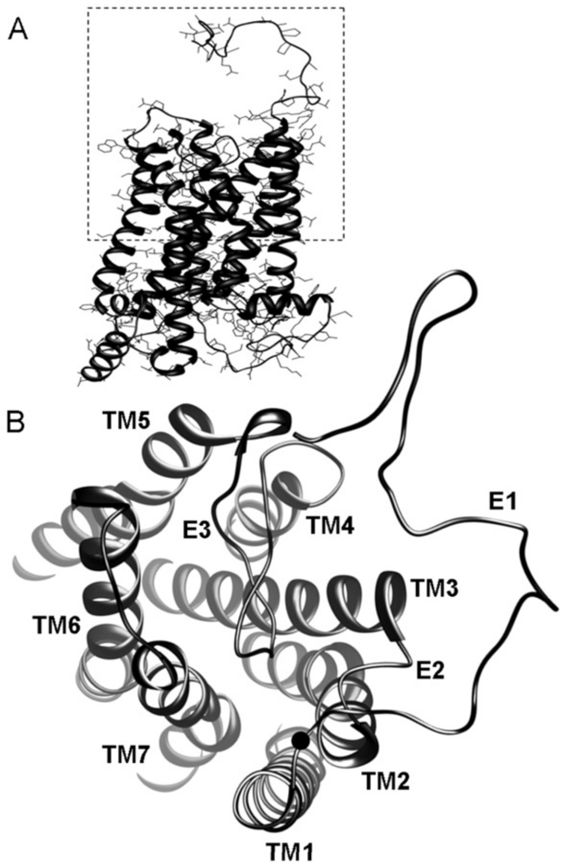

Fig. 3. Model of the human oxytocin receptor. (A) Side view of the receptor structure. The rectangle mark the section shown in panel B from the top. (B) Extracellular view (i.e. rotation by 90° out of the plane) of the marked section in A. The transmembrane helices are arranged in an anti-clockwise fashion around the central pocket site. Presumably, receptor activation is accompanied by a reduction in the bend of helix 6. Agonists and antagonists bind at different sites around the central pocket site. Transmembrane helices 4 and 5 are possibly involved in homodimeric receptor association. Small black circle, position of R34 involved in oxytocin binding. The model was constructed by homology modelling using the programme 'ESyPred3D' with the 3D structure of rhodopsin (pdb code 1f88) as template (Lambert et al., 2002). The UCSF Chimera programme (www.cgl.ucsf.edu/chimera) was employed for graphical display.

Receptor activation

A network of interhelical hydrogen bonds and hydrophobic interactions are proposed to constrain the receptor in the inactive conformation. During activation of GPCRs rotations of transmembrane helices relative to each other are proposed to occur. In particular, due to bend reduction helix 6 may move away from helix 3 and, at its cytoplasmic end, towards helix 5 (Ghanouni et al., 2001). However, recent crystallographic studies with photoactivated rhodopsin (albeit not at the highest resolution) surprisingly suggested that receptor activation is accompanied by only minor structural rearrangements of transmembrane helices and their orientation to each other (Salom et al., 2006). Instead, the activated receptor undergoes structural disordering (relaxation) mainly at the extracellular loops i2, i3 and the C-terminus. This subsequently leads to increased binding of the heterotrimeric G protein and its activation.

OTRs are able to couple to at least $G\alpha_q$ and $G\alpha_i$ proteins (Ku et al., 1995; Strakova and Soloff, 1997) that stimulate together with $G_{\beta\gamma}$ the phospholipase C-β isoforms. This can induce an increase in intracellular calcium concentration, activation of various protein kinases (e.g. mitogen-activated protein kinases, protein kinase C, myosin light-chain kinase) and/or production of prostaglandin E2 (Gimpl and Fahrenholz, 2001; Zingg and Laporte, 2003). In dependence on G protein coupling, OTRs can transduce growth-inhibitory or proliferatory signals. Receptor coupling to $G\alpha_i$ was shown to inhibit cellular proliferation whereas coupling to another Gα protein was able to induce a growth-promoting effect. Interestingly, atosiban can act as a functional selective ligand, antagonistic on receptor-$G\alpha_q$ coupling but as an agonist on receptor-$G\alpha_i$ coupling (Reversi et al., 2005). More or less all intracellular loops may contribute to the coupling selectivity of the OTR. For coupling to G_q proteins, two regions at the cytoplasmic side of the OTR have been found to harbour critical motifs: juxtamembrane regions of intracellular loop 3 (notably residue K270) and the C-terminus (see dotted boxes in Fig. 2) (Hoare et al., 1999; Zhong et al., 2004, 2007). Particularly, residues in the hydrophilic face of the putative amphipathic helix 8 of the OTR may be important for receptor

conformation and $G\alpha_q$ coupling (Zhong et al., 2004). The extent to which OTRs signal via G_i seems to be species-specific, as judged by sensitivity against pertussis-toxin (Blanks et al., 2007). Following stimulation of the OTR, the homologous desensitization process is initiated by receptor phosphorylation and subsequent arrestin association. Rapid and transient phosphorylation by GPCR kinase-2, protein kinase C-α and arrestin binding has been demonstrated in heterologous expression systems (Berrada et al., 2000; Hasbi et al., 2004). Internalization of the OTR primarily occurs via the clathrin-pathway (Smith et al., 2006).

Structural studies with rhodopsin favour a model in which dimers or higher-order oligomers are functional receptor states (Park and Palczewski, 2005). Using atomic force microscopy, rhodopsin is the only receptor whose native oligomeric arrangement has been revealed (Fotiadis et al., 2003). The packing arrangement of rhodopsin molecules in native disk membranes appears to possess not only intradimeric, but also interdimeric and row–row contacts. Thereby, the strongest interactions are provided by homodimeric receptor contacts via the transmembrane segments 4 and 5 (Fotiadis et al., 2006). Some evidence is provided that OTRs are also present in form of dimeric or oligomeric complexes at the cell surface (Terrillon et al., 2003; Devost and Zingg, 2003). On the other hand, a recent study convincingly demonstrated that the monomeric β_2-adrenergic receptor is sufficient to activate its corresponding G protein (Whorton et al., 2007).

Oxytocin receptors and cholesterol

One remarkable property of the OTR concerns its functional dependence on cholesterol and divalent cations. In vitro and in vivo, the receptor can exist in at least two affinity states for agonists and antagonists. To exist in high-affinity state, the receptor requires at least two factors, namely divalent cations such as Mn^{2+} or Mg^{2+}, and a high-cholesterol environment. In dependence on these factors, the receptor can reversibly change its conformation from high- ($K_d \sim 1\,nM$) to low-affinity ($K_d \sim 100\,nM$) and vice versa (Gimpl et al., 1995; Klein et al., 1995; Gimpl et al., 1997). Unfortunately, the low-affinity sites are difficult to characterize with binding studies. So, it cannot be excluded that more than one population of low-affinity receptor states exist. In each case, the interaction of cholesterol with OTRs is of high specificity and not due to mere changes of the membrane fluidity (Gimpl et al., 1997). To explore the cholesterol-binding domain of the OTR, a variety of mutagenized or chimeric receptor constructs have been analysed. Site-directed mutagenesis was performed on the basis of a molecular modelling study (Politowska et al., 2001). Therein, cholesterol molecules were docked into the OTR using favourable and non-functional cholesterol analogues. Residues suggested as critical for interaction with cholesterol are marked in Fig. 2. Unfortunately, mutagenesis of most of these residues to alanin resulted in receptors with nearly undetectable expression yield (unpublished). We hypothesize that the OTR requires cholesterol interaction not only to support its high-affinity state but also for efficient receptor expression. This conclusion is supported by studies with insect cells. When OTRs are expressed in insect cells that naturally possess a low cholesterol content, addition of cholesterol to the culture has a much stronger effect to gain high-affinity state expression than addition of the same cholesterol amounts to plasma membranes (Gimpl et al., 1995). Possibly, cholesterol supports protein folding or supports efficient vesicular transport through the secretory pathway. In a second approach, chimeric OT/cholecystokinin B receptor constructs were created. This strategy was used in view of our observations that the cholecystokinin B receptor showed no specific cholesterol interaction but behaved similar to the OTR with respect to signal transduction (Gimpl et al., 1997). Preliminary results with the chimeric receptor constructs showed that transmembrane segments 6 and 7 are dispensable for cholesterol binding.

Cholesterol also stabilizes the OTR against thermal or proteolytical degradation (Gimpl and Fahrenholz, 2002). In cholesterol-rich microdomains such as caveolae or lipid rafts, OTRs were significantly more stable than in cholesterol-poor domains (Gimpl and Fahrenholz, 2000). If the

Table 1. The effect of cholesterol on the ligand-binding parameters of some GPCRs

Receptor	Affinity[a]	Capacity[a]	References
Cannabinoid: CB_1	None	−	Bari et al. (2005)
Cholecystokinin: CCK_2	None	+	Gimpl et al. (1997)
Galanin: GAL_2	+	None	Pang et al. (1999)
5-Hydroxytryptamine: $5\text{-}HT_{1A}$	+	+	Pucadyil and Chattopadhyay (2004); Pucadyil et al. (2005)
5-Hydroxytryptamine: $5\text{-}HT_7$	None	+	Sjogren et al. (2006)
Metabotropic glutamate: $mGlu_1$	+	n.d.	Eroglu et al. (2003)
Opioid: δ	+	None	Huang et al. (2007)
Opioid: κ	+ Or none[b]	None	Xu et al. (2006)
Oxytocin	+ +	None	Gimpl et al. (1997)
Tachykinin: NK_1	None	None	Monastyrskaya et al. (2005)

[a]Positive correlation (i.e. cholesterol supporting high affinity or capacity) denoted with '+', negative correlation (e.g. cholesterol depletion increases capacity or affinity) denoted with '−'; n.d., not determined.
[b]'+' For a partial agonist, 'none' for an antagonist.

OTR becomes forced into lipid rafts by C-terminal fusion with caveolin, a marker of these domains, a switch in the proliferative behaviour of cells has been observed: a growth-inhibitory effect induced by OT reversed to a proliferative response (Guzzi et al., 2002). Thus, the population of OTRs residing in cholesterol-rich domains may transduce via different signalling cascades as compared to receptors localized in domains with a lower cholesterol amounts. The integrity of cholesterol-rich domains also influences the behaviour and signalling of many other GPCRs (Chini and Parenti, 2004). A couple of GPCRs have now been analysed with respect to their ligand-binding parameters in dependence on the cholesterol status of the cells or membranes. For almost all receptors so far studied, cholesterol is positively correlated with the affinity state or ligand capacity (Table 1).

Oxytocin receptor research: quo vadis?

The major goal of receptor biochemistry is to obtain structural informations about the receptor, e.g. their conformational changes induced by ligand binding or G protein association. Despite much efforts purification has been achieved only for a handful of receptors. In case of the OTR, all attempts to purify the functional receptor have been unsuccessful to date. However, even when receptor purification would be successful, uncertainty remains whether structural informations obtained with purified preparations truly reflect the situation in vivo. This is due to the fact that the solubilization process dramatically alters the lipid environment of the purified receptor. A receptor that is normally localized in a specific microdomain, e.g. in a cholesterol-rich milieu for the OTR, might behave differently when isolated from its lipid environment. One possibility to overcome these limitations is to perform studies with receptors that are tagged with different reporter groups via recombinant DNA techniques. The most prominent and widely used reporter is green fluorescent protein (GFP) and its various spectroscopic variants. The OTR tagged at the C-terminus with GFP behaves like the wild-type receptor. Using fluorescence microscopy or spectrofluorometry receptor trafficking could be followed and/or total receptor amounts could be quantitated independent on their affinity state (Gimpl and Fahrenholz, 2000). In case of the parathyroid hormone receptor and the α_{2A}-adrenergic receptor even two GFP variants have been introduced (at the intracellular loop 3 and the C-terminus, respectively) that allowed to measure the millisecond activation switch of the receptors by fluorescence resonance energy transfer (FRET) (Vilardaga et al., 2003). However, GFP is a relatively large molecule. Most likely, it will perturb receptor function when introduced for example at positions within loop domains of the receptor. Ideally, the researcher wishes to introduce small reporter groups into different positions of the

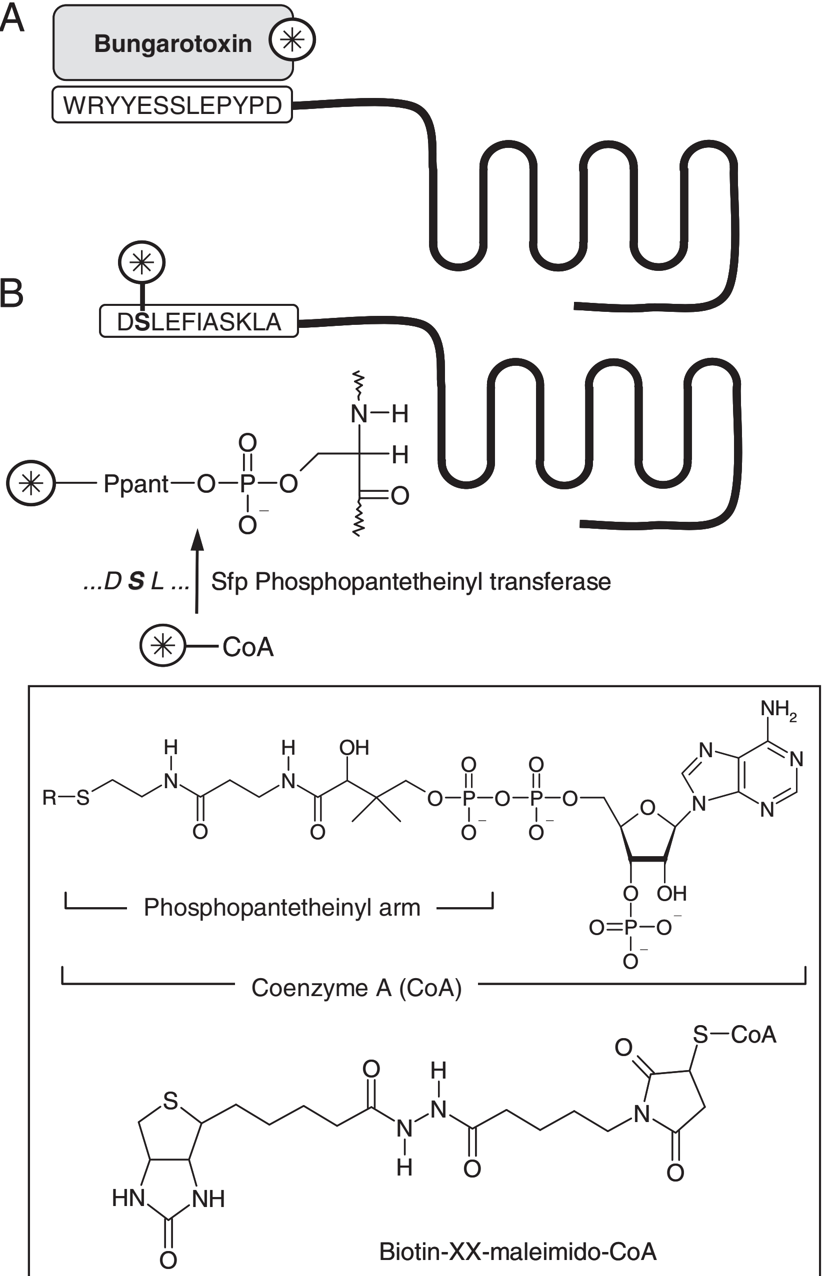

Fig. 4. Strategies for site-specific receptor labelling in living cells. (A) Bungarotoxin specifically recognizes the indicated oligopeptide tag fused here to the N-terminus of the receptor. The reporter group (encircled asterisk) is covalently attached to bungarotoxin. (B) The indicated 11-aa (ybbR) tag is covalently labelled with a reporter group (encircled asterisk) that has been introduced by Sfp phosphopantetheinyl (Ppant) transferase. This enzyme accepts a wide range of reporter-CoA derivatives as substrate and transfers the reporter group onto a serin residue within the tag. The inset shows the structure of biotin-XX-maleimido-CoA (XX denote a spacer element) as an example of a substrate used for the labelling of the oxytocin receptor.

receptor in a highly selective manner. The methodology for site-specific labelling of proteins has greatly expanded in the last years. Here, we will shortly describe two promising tagging strategies that both use small peptide targeting sequences: (i) the 13-aa α-bungarotoxin-binding site (BBS) (Fig. 4A), and (ii) a 11-aa tag (named 'ybbR'-tag because part of its sequence is derived from the ybbR ORF in the *B. subtilis* genome) for covalent attachment of reporter groups from CoA-derivatives (Fig. 4B).

In the first approach, the BBS tag binds to the ~8.4 kDa protein bungarotoxin with high-affinity (Sekine-Aizawa and Huganir, 2004). Due to the possibility to attach different reporter groups (e.g. biotin or fluorochroms) to bungarotoxin, this strategy offers a versatile tool to explore receptor trafficking. We have demonstrated that this strategy principally works for the OTR (unpublished). Notably, only receptors residing at or translocating to the plasma membrane can be selectively labelled simply by adding fluorescent bungarotoxin to the culture medium. This is a major advantage as compared to the GFP-tagged receptors that are always present in all stages throughout the secretory pathway. The second approach exploits the property of Sfp phosphopantetheinyl (Ppant) transferase that covalently transfers 4′-phosphopantetheinyl groups from CoA-derivatives onto the serin residue within the 'ybbR'-tag (Yin et al., 2005). The Sfp transferase accepts a wide range of small molecules attached to CoA as substrate. Thus, the method allows the covalent labelling of ybbr-tagged receptors by a variety of reporter probes such as biotin, photoreactive compounds, fluorophores or small peptides. The site-specific labelling reaction can be performed on cell lysates or living cells within minutes. Application of this method is more laborious because it requires the synthesis of CoA-containing substrates and the purification of the Sfp transferase. Provided that the tag adopts an amphiphilic α-helix within its targeting sequence of the receptor, a covalent labelling with the reporter group may be achieved. We are currently testing this strategy for the OTR.

The employment of environmental-sensitive fluorescent probes in combination with orthogonal receptor labelling might enable us in the near future to measure conformational changes of the receptor by spectroscopic and imaging techniques in living cells.

Abbreviations

BBS	bungarotoxin-binding site
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
OT	oxytocin
OTR	oxytocin receptor
Ppant	phosphopantetheinyl
Sfp	surfactin production genetic locus from *B. subtilis*

Acknowledgements

We thank Christa Wolpert (Mainz) for technical assistance, Dr. Torsten Reinheimer (Ferring Pharmaceuticals, Copenhagen, Denmark) for cooperative support, Dr. Christopher T. Walsh (Harvard Medical School, Boston, USA) and Dr. Jun Yin (University of Chicago, USA) for the supply of the Sfp posphopantetheinyl transferase cDNA.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 18

Opposite effects of oxytocin and vasopressin on the emotional expression of the fear response

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Abstract: Oxytocin and vasopressin are two neuropeptides that have been extensively studied for their systemic and physiological roles. Studies in rodents show that oxytocin and vasopressin play an opposite role in several behavioural and physiological tests for anxiety and fear. Their effects on single cell activity in the central amygdala (CeA) triggered a number of electrophysiological studies that allowed us to develop a model of their opposing effects. In our model, GABAergic neurons in the lateral part of the central amygdala are excited by oxytocin and project to the medial part where they inhibit neurons that can be excited by vasopressin. Besides oxytocin and vasopressin, the CeA expresses a large number of other neuropeptide receptors and the question arises if a similar model can apply to their actions. We here develop a hypothesis in which neuropeptides, through their effects on distinct populations in the CeA, affect specific projections and specific physiological expressions of the fear response. Our hypothesis may be of importance for the current interest in neuropeptide receptors as therapeutic targets for neuropsychiatric disorders.

Keywords: amygdala; fear; anxiety; central amygdala; neuropeptide; bed nucleus of stria terminalis; oxytocin; vasopressin

Introduction

Oxytocin and vasopressin in the limbic system

Oxytocin and vasopressin are two structurally related nonapeptides that differ only in the third and eighth positions. They form a ring structure through a disulphide bridge connecting two cysteine residues. Both are predominantly synthesized in magnocellular and parvocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus (Swaab et al., 1975; Hou-Yu et al., 1986), but are also found in other regions of the brain. Three different receptors for vasopressin have been described until now, V1a, V1b and V2 receptors, as well as one oxytocin receptor. Vasopressin receptors are selective for vasopressin, whereas oxytocin receptors recognize not only oxytocin, but also vasopressin with a lower affinity. Their effects are mediated by an intracellular mechanism coupled to a G protein. More specifically, V1a and V1b receptors act via

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DOI: 10.1016/S0079-6123(08)00418-4

hydrolysis of phospho-inositol and activate intracellular calcium, while the V2 receptor activates adenylate cyclase and cAMP, although some motoneurons appear to use different signalling pathways (Reymond-Marron et al., 2006). Activation of the oxytocin receptor typically leads to phospholipase C activation, mobilization of calcium and stimulation of phosphatidyl inositol (Kimura and Saji, 1995). Receptors for oxytocin and vasopressin have been found in many areas of the brain, including the limbic system.

Initial studies in rodents seemed to indicate that vasopressin could increase memory (de Wied et al., 1976), antagonize amnesia (Bookin and Pfeifer, 1977) and facilitate memory consolidation (Kovacs et al., 1979; de Wied, 1980). A cellular basis for these effects arose with the discovery that inhibitory neurons in the CA1 area of the hippocampus could be directly excited by oxytocin or vasopressin, while pyramidal neurons were indirectly inhibited (Muhlethaler et al., 1982, 1984; Tiberiis et al., 1983; for review, see Raggenbass, 2001). Oxytocin and its structural analogues were more potent than vasopressin, indicating that the peptide effects were mediated by oxytocin receptors (Muhlethaler et al., 1984). These findings were followed by clinical studies in patients affected by memory problems. While some studies showed an improvement in memory mediated by vasopressin (Weingartner et al., 1981; Laczi et al., 1982), others reported mixed findings (Guard et al., 1986) or were unable to show positive results (Durso et al., 1982; Jennekens-Schinkel et al., 1985), and several critical reviews on the vasopressin-memory theory followed (Sahgal, 1984; Strupp and Levitsky, 1985).

In recent years, a number of studies have appeared describing a role for vasopressin and oxytocin in the regulation of the emotional expression of fear. Their effects appeared to be localized to the amygdala, an almond-shaped area in the brain immediately anterior to the ventral hippocampus. The amygdala plays a crucial role in fear responses, and synaptic plasticity in this structure directly underlies an emotional form of memory called "fear-conditioned learning". It is the intention of this review to summarize these findings and indicate a number of open questions and perspectives that they evoke.

The role of the amygdala in the physiological expression of fear

Fear and anxiety are normal reactions to danger and are not in themselves a manifestation of pathological disorders. Under normal circumstances, fear can positively contribute to "fear-conditioned learning", a process during which a relation is established between a harmful and neutral stimulus. Recently, it has become clear that "fear-conditioned learning" involves a potentiation of synaptic transmission in the basolateral part of the amygdala (BLA; for review, see LeDoux, 2000). The BLA sends projections to the central amygdala (CeA), which projects further to the hypothalamus and brain stem. Through the projections to the autonomic centres, the CeA affects the physiological expression of fear. By activating the CeA, the fear response leads to a potentiated acoustic startle, freezing, hypoalgesia, elevated blood pressure and heart rate, increased respiratory rhythm and intestinal motility (Killcross et al., 1997; Maren, 1999).

The CeA is typically depicted as consisting of four subdivisions: the central medial, lateral, intermediate and capsular parts (Jolkkonen and Pitkanen, 1998). The medial part of the central amygdala (CeM) plays a particularly important role as an interface for the physiological expression of fear, because it receives projections from the BLA and forms the main output from the amygdala to the brainstem (Hopkins and Holstege, 1978; Veening et al., 1984). The CeM appears to be under the control of the lateral and capsular part of the central amygdala, which we will refer to together as "CeL". Thus, the CeL sends inhibitory (GABAergic) projections to the CeM, and can act as a "gain control", modulating the CeM activity that is triggered by the input from the BLA (Petrovich and Swanson, 1997; Jolkkonen and Pitkanen, 1998). Indeed, from behavioural experiments, it appears that changes in synaptic transmission in the CeA may play an important role in the behavioural responses related to fear and anxiety (see below).

In recent years, awareness has grown that the amygdala is part of a larger complex, called the "extended amygdala". The extended amygdala includes several structures that are highly

homologous to the amygdala and that are connected via the stria terminalis. One of these is the anteriorly located bed nucleus of the stria terminalis (BNST). While the CeA mediates conditioned fear reactions activated by relatively short stimuli in highly predictable situations, the BNST has been suggested to mediate unconditioned fear responses to relatively long, contextual cues under conditions in which the perceived danger is not highly predictable and requires a sustained state of defensive preparedness (Davis et al., 1997; Davis, 2006). It still remains to be assessed to what extent similar local inhibitory projections as in the CeA can also be found in the BNST.

Neuropeptidergic modulation of fear in the amygdala

Factors that modulate GABAergic projections from the CeL to the CeM may be expected to importantly influence the autonomic fear response. Indeed, various substances that could modulate local inhibition in the CeA have been shown to influence fear behaviour. Among these, the benzodiazepines are well known for their anxiolytic actions by enhancing the postsynaptic GABA(A) receptor responses to GABA. Their effects are mediated through the α2 subunit of the GABA(A) receptor (Low et al., 2000), which is expressed in the CeA (Pirker et al., 2000; Kaufmann et al., 2003) and is responsible for its local effects (Kang-Park et al., 2004). Furthermore, various neuropeptides whose receptors are expressed in the CeA are well known for their effects on anxiety and fear behaviour. Among these are corticotropin-releasing hormone (CRF) known for its anxiogenic effects, and neuropeptide Y (NPY) known for its anxiolytic effects (Heilig et al,. 1994). Evidence for the presence of CRF (Van Pett et al., 2000) and NPY (Parker and Herzog, 1999) receptors has indeed been found in the CeA.

Moreover, various behavioural studies have implicated oxytocin and vasopressin in the modulation of anxiety and fear responses. Oxytocin has anxiolytic and antistress effects (McCarthy et al., 1996; Bale et al., 2001; Ring et al., 2006), facilitates pair-bonding and decreases maternal fear leading to enhanced mother–infant interactions (Fleming and Corter, 1988; Rosenblatt, 1994; Uvnas-Moberg et al., 1994; Uvnas-Moberg, 1997; Stern, 1997; Champagne and Meaney, 2001). Vasopressin, on the other hand, causes increased fear responses as reflected by a decrease in heart rate (Roozendaal et al., 1993), increases in colonic motility during an emotionally stressful situation (Bueno et al., 1992) and causes increases in anxiety-like behaviour (Koolhaas et al., 1998; Everts and Koolhaas, 1999; Landgraf, 2006).

The involvement of the CeA in these behavioural effects was initially shown by local injections of vasopressin and oxytocin: vasopressin caused an increased fear response as reflected by a decrease in heart rate and behavioural motility, while oxytocin led to an increase in heart rate, behavioural motility and to higher corticosterone levels thereby indicating a decreased fear response (Roozendaal, 1992a, 1993). Different projections of vasopressin and oxytocin receptive cells to behavioural and physiological output systems were proposed to be at the basis of these opposite effects (Roozendaal et al., 1993). In this context, it was of interest that the CeA shows a clear complementary expression of oxytocin and vasopressin receptors (Tribollet et al., 1988; Veinante and Freund-Mercier, 1997). In CeM, vasopressin receptors are highly expressed, while oxytocin receptors are found in the CeL. This complementarity can be found throughout the extended amygdala, persisting up to the BNST (Veinante and Freund-Mercier, 1997). These findings, in combination with the GABAergic projections from the CeL to the CeM subdivision (Petrovich and Swanson, 1997; Jolkkonen and Pitkanen, 1998), suggested a different cellular mechanism underlying the opposite effects of oxytocin and vasopressin in the CeA. They raised the possibility that oxytocin receptors in the CeL and vasopressin receptors in the CeM could affect respective pre- and postsynaptic elements of the local GABAergic circuitry.

Effects of oxytocin and vasopressin on synaptic transmission in the central amygdala

Condes-Lara et al. (1994) and Lu et al. (1997) were the first to show effects of oxytocin and vasopressin on cellular activity in the CeA. These

studies employed extracellular recordings techniques and showed potent, reversible and specific effects of both neuropeptides throughout the CeA. They laid the basis for a series of investigations in our laboratory concerning the precise role of oxytocin and vasopressin in the different parts of the CeA (Huber et al., 2005). Using extracellular single-unit recordings, we were able to identify two major populations of neurons, one excited by vasopressin but inhibited by oxytocin, the other only excited by oxytocin and unresponsive to vasopressin. The effects of vasopressin were mediated by the V1a receptor, while those of oxytocin were blocked by traditional oxytocin receptor antagonists. The inhibitory effects of oxytocin could be reduced by blocking GABA-ergic transmission, which suggested that they might be indirectly mediated by an increased release of GABA. The excitatory effects, on the other hand, were unaffected by GABA and appeared to be directly mediated, similarly to the findings of Terenzi and Ingram (2005).

To investigate the morphology and projections of these excited neurons, we combined sharp electrode recordings with intracellular labelling. These revealed that oxytocin-excited neurons were localized in the CeL, whereas vasopressin-excited cells were found in the CeM. Subsequent tracing studies showed that the axon collaterals of the oxytocin-excited cells projected far into the CeM, and immunohistochemical staining showed that they were GABAergic. This suggested further to us that the inhibitory effects of oxytocin might be synaptically mediated by an increased release of GABA. We therefore examined whether oxytocin could enhance release of GABA from these CeL neurons that project to the CeM.

Using whole-cell patch-clamp recordings in the CeM, we could demonstrate that the inhibitory effects of oxytocin were indeed related with a massive increase of inhibitory GABAergic currents, induced by the activation of the CeL neurons. We subsequently tested the relevance of this mechanism for synaptic transmission in the amygdala by measuring oxytocin and vasopressin effects on evoked neurotransmission release. These findings confirmed that vasopressin increased and oxytocin decreased the probability of evoking action potentials in the CeM following stimulation of BLA afferents. Oxytocin, on the other hand, was able to enhance the probability of evoking action potentials in neurons of the CeL, following stimulation of their specific afferents (Huber et al., 2005).

A model for the opposing effects of vasopressin and oxytocin

The above findings led us to the development of a model in which the opposing behavioural effects of vasopressin and oxytocin are caused by a selective activation of two distinct populations of neurons in the GABAergic network of the CeA. In our model, GABAergic neurons in the CeL are activated by oxytocin and project to the CeM, where they exert their inhibitory effects on neurons that are directly activated by vasopressin receptors (Fig. 1). The oxytocin-excited neurons thus form a major inhibitory projection from the CeL onto the CeM nucleus. In view of the fact that the CeM receives the main excitatory input from the basolateral complex, oxytocinergic modulation of the inhibitory projection from the CeL onto the CeM could therefore act as a "gain control" for the processing of this input and the subsequent output from the CeA (Huber et al., 2005).

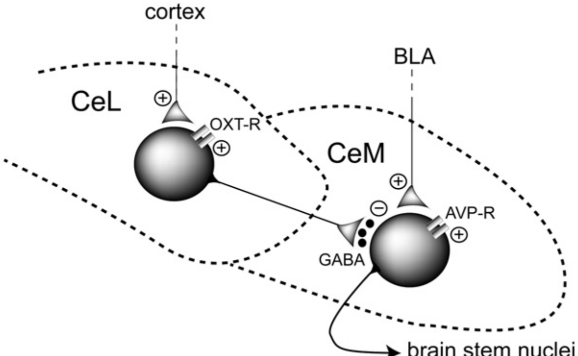

Fig. 1. Circuitry underlying the opposite effects of oxytocin and vasopressin characterized by local inhibitory projections from the CeL to projections neurons in the CeM. Oxytocin acts via oxytocin receptors (OXT-R) expressed on neurons in the CeL, while vasopressin activates vasopressin receptors (AVP-R) in the CeM (for further explanations see text).

What are the consequences of this modulation by oxytocin and vasopressin for the expression of anxiety and fear behaviour? Differences in expression of vasopressin and oxytocin or their receptors may be related to individual variabilities in fear and anxiety phenotypes. For example, the Swiss sublines of Roman high avoidance (RHA) rats have been selected for less emotional behaviour than the low avoidance (RLA) rat strain (Driscoll et al., 1998; Steimer and Driscoll, 2003). As it turns out, injections of vasopressin and oxytocin in the CeA of RHA rats are without any effect, while in RLA rats they have opposite effects as described for normal rat strains (Roozendaal et al., 1992b). Similarly, in Wistar rats bred for either high (HAB) or low (LAB) anxiety-related behaviour, the extreme trait anxiety in HAB rats has been related with a polymorphism in the promotor structure of the vasopressin gene (Landgraf et al., 2007). While it is possible that these different behavioural phenotypes are directly related to changes in vasopressin and oxytocin signalling in the CeA, other modulating factors may also be involved. For example, in RHA and RLA rats, the CRF system of the CeA appears to differentially affect cardiovascular and behavioural responses (Wiersma et al., 1998) and RLA rats have significantly more CRF neurons in the CeA than RHA rats (Yilmazer-Hanke et al., 2002). Interestingly, maternal care has been shown to both affect oxytocin and vasopressin receptor expression as well as anxiety and fear behaviour in adulthood, pointing further to a causal relation between behaviour and neuropeptidergic signalling (Francis et al., 2002).

Human studies

As mentioned above, the initial findings of vasopressin effects on explicit memory have triggered at the time a number of clinical studies, but with moderate success. One of the constraints with human behavioural studies is the targeting of neuropeptides to the brain, and it has been shown that oxytocin and vasopressin do not or hardly cross the blood–brain barrier (Ermisch et al., 1985). Obviously, local intracerebral injections cannot be performed in humans and need to be replaced by other methods. The application of neuropeptides in the form of a spray has been the method of choice in most studies. Indeed, this approach appears able to deliver different types of neuropeptides to the human brain (Born et al., 2002).

Using oxytocin applied by spray, Kosfeld et al. (2005) recently reported how its application can increase mutual trust in human volunteers. If these effects of oxytocin are the result of an inhibition in the CeA, their results are consistent with previous findings that showed an increase in human trust following damage to the amygdala (Adolphs et al., 1998). Effects of oxytocin on amygdala activation have been subsequently reported by Kirsch et al. (2005). In a functional magnetic resonance imaging (fMRI) study, this group showed that intranasal administration of oxytocin reduces amygdala activation and modulates its participation in functional networks activated by processing of fearful visual stimuli that reliably engage the amygdala. Compared to placebo, oxytocin significantly decreased amygdala activation.

Few studies until now have addressed the effects of vasopressin in the human amygdala. A currently promising candidate with antidepressant and apparently also anxiolytic proprieties is the V1b antagonist SSR149415 (Serradeil-Le Gal et al., 2002). Its antidepressant site of action has been related with the expression of the V1b receptor in the lateral septum (Stemmelin et al., 2005), while an anxiolytic site of action has been proposed for its blocking effects in the BLA (Salome et al., 2006). In view of these findings in the BLA and previous findings in the CeA, it is possible that antagonists for the vasopressin receptor may present promising candidates for new anxiolytic treatments.

Open questions

The above findings suggest an important endogenous function for vasopressin and oxytocin in the CeA. Indeed, local injections of these peptides affect anxiety and fear behaviour, and variations in their expression level can be related with differences in behaviour. What still remains largely unknown is the precise origin of endogenous

vasopressin and oxytocin in the amygdala. Vasopressin expression has been found outside of the paraventricular and supraoptic nucleus of the hypothalamus, among others in the medial amygdala (MeA) and BNST (Caffe and van Leeuwen, 1983; van Leeuwen and Caffe, 1983). It is possible that, through projections from the stria terminalis or by local dendritic release from the MeA, vasopressin reaches its receptors in the CeA. Neuropeptides such as oxytocin and vasopressin may diffuse over long distances in the extracellular fluid (Landgraf and Neumann, 2004). While the exact origin of the oxytocin released in the CeA remains to be determined, it appears to be under dynamic control, as stressors such as forced swimming has been shown to increase its endogenous release in male rats (Ebner et al., 2005).

Another question concerns the potential actions of oxytocin and vasopressin in other nuclei of the amygdala. As mentioned above, the amygdala is part of a larger complex, called the "extended amygdala" and the complementary receptor expression for oxytocin and vasopressin in the CeA can be found throughout this complex, extending to the BNST (Veinante and Freund-Mercier, 1997). Indeed, recent findings have shown excitatory responses to oxytocin in the lateral part of the BNST, in a part homologous to the CeL. Moreover, it appears that oxytocin also affects activity in the MeA and its homologous medial part of the BNST (Terenzi and Ingram, 2005; Wilson et al., 2005). The precise roles of oxytocin and vasopressin in these areas remain to be assessed. Recent findings have suggested a function for vasopressin and oxytocin in the amygdala in aggressive behaviour (Blanchard et al., 2005; Ferris, 2007; Veenema and Neumann, 2007; Wersinger et al., 2007), and this could be a promising field of research that is still relatively unexplored.

Towards a new hypothesis for neuropeptide function in the central amygdala

The regions of the brain implicated in the modulation of the anxiety and fear responses are well studied nowadays. It is known that the amygdala plays a crucial role in the triggering of the fear reaction (Hitchcock and Davis, 1986; Davis, 1992) and by virtue of its projections to various brain stem nuclei, these reactions can be expressed as changes in a number of physiological parameters (Zhao and Davis, 2004; Paré et al., 2004). What remains rather unexplored is how these projections are precisely organized and which specific neuronal populations in the CeA are at their origin.

The CeA projects to a large number of nuclei in the brain stem that mediate the physiological expression of fear. Among these are the periaqueductal grey (PAG), located around the central canal of the brainstem, that regulates among others freezing behaviour (Behbehani, 1995), the nucleus of the solitary tract (NTS), the dorsal motor nucleus of the vagus (DMN) and the rostral ventrolateral medulla (RVLM, Fig. 2). The NTS is the major visceral sensory relay nucleus in the brainstem and forms with the DMN the so-called dorsal vagal complex (DVC). Its medial two-thirds is probably the sole source of parasympathetic neurons that innervate the upper gastrointestinal tract in a visceral topographical manner and mediates effects of amygdala activation on the gastrointestinal system (Loewy and Spyer, 1990). The RVLM, on the other hand, appears to play an important role in the cardiovascular, respiratory and papillary responses evoked by stimulation within the CeA (Hilton and Zbrozyna, 1963; Hilton et al., 1983). While the above nuclei receive projections only from the CeM, the parabrachial nucleus is innervated by both the CeL and CeM. This nucleus, localized around the superior cerebellar pendumcle, receives neurotensin, somatostatin and CRF containing projections from the CeA (Moga and Gray, 1985) and is thought to be important for pain regulation as well as for respiratory responses (Harper et al., 1984; Masaoka and Homma, 2004).

We currently do not know whether oxytocin and vasopressin affect projections to all these brainstem nuclei or if they modulate just a specific subset. Our previous findings have shown that only about 50% of all spontaneously active cells in the CeA were excited by vasopressin and that of

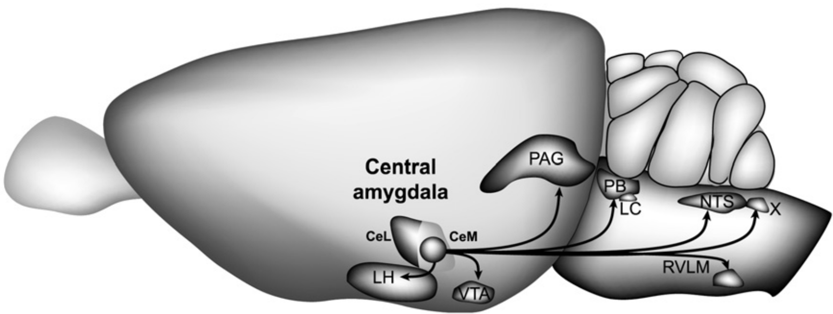

Fig. 2. Projections from the central amygdala. Lateral (CeL) and medial (CeM) part of the central amygdala, lateral hypothalamus (LH), ventral tegmental area (VTA), periaqueductal grey (PAG), parabrachial nucleus (PB), locus coeruleus (LC), nucleus of the solitatary tract (NTS), dorsal motor nucleus of the vagus (X) and rostral ventrolateral medulla (RVLM).

these about half were inhibited by oxytocin (Huber et al., 2005). In this context, it becomes of special interest that, besides oxytocin and vasopressin, the CeA expresses receptors for many other neuropeptides. Among these are CRF, NPY, opioids, substance P, calcitonin gene-related peptide, neurotensin, cholecystokinin, thyrotropin-releasing hormone (TRH), orexin, galanin, bombesin, somatostatin, vasoactive intestinal polypeptide, angiotensin II and atrial natriuretic peptide. In view of this multitude of receptors, it is possible that there are other other ''neuropeptidergic couples'' that can influence the output from the CeA in an opposite manner, similar to oxytocin and vasopressin. If this is the case, we could imagine that these act on a subset of neurons with brain stem projections that are not modulated by vasopressin and oxytocin. Thus, we could hypothesize that different neuropeptides in the CeA can specifically affect distinct neuronal populations and thereby regulate specific physiological expressions of fear.

What is the current evidence in favour of such a hypothesis? It would, first of all, imply that target brain stem nuclei should be innervated by distinct neuronal populations within the CeA. Thompson and Cassell (1989) showed distinct populations in the CeM at the origin of projections to the NTS and the RVLM and suggested that neurons projecting to the RVLM specifically contain galanin. Other groups have shown that different areas within the DVC are innervated by axons originating from distinct regions within the CeA (Veening et al., 1984; Danielsen et al., 1989; Liubashina et al., 2000; Zhang et al., 2003). Through this pathway, electrical stimulation of the CeM leads to an increase in gastric acid secretion and rapid ulcer formation (within 4 h, Innes and Tansy, 1980; Henke et al., 1991) and an inhibition of gastric motility, while stimulation of the CeL causes an excitatory effect (Liubashina et al., 2002). Saha et al. (2000, 2002) have shown that the caudal cardiovascular region of the NTS expresses somatostatin-2A receptors that receive specific innervations from somatostatin expressing neurons in the CeA. It thus appears that at least to a certain extent, separate populations in the CeA affect different autonomic expressions of fear.

In addition, this hypothesis would imply that neuropeptides can be assigned specific functions in the CeA. Currently, there are only few indications of neuropeptides acting on specific physiological aspects of the fear response. Among these, injection of neurotensin in the CeA was found to decrease gastric motility and ulcers (Henke et al., 1988), but to have no effects on heart rate or blood pressure (Brown and Gray, 1988). Furthermore, in vivo application of angiotensin in the CeA leads to an increase in blood pressure, but a decrease in heart rate (Brown and Gray, 1988). Interestingly, the relative number of the angiotensin-excited cells in the CeA was found to vary between rat strains and to be lower in a high-anxiety, hypertensive rat strain (Albrecht et al., 2000), similarly to receptor expression

differences for vasopressin and oxytocin found in RHA/RLA and HAB/LAB rats.

These findings bring up the interesting possibility that, according to genetic background and upbringing, each individual develops its particular combination and expression levels of neuropeptide receptors on subsets of neuronal populations in the CeA. It becomes possible that distinct expression levels could represent the basis of individual differences in emotionality or in differences in expression of anxiety disorders. Such differences may, for instance, underlie different types of panic disorders, as determined by the effects of a panic attack on the respiratory or cardiovascular system (Buller et al., 1986; Bovasso and Eaton, 1999).

Conclusions and perspectives

Recent research has brought forward a model to explain the opposite effects of vasopressin and oxytocin on the physiological expression of fear through local actions on an inhibitory circuit in the CeA. Their opposing actions may apply throughout the extended amygdala and may also serve to describe a general mechanism underlying opposite effects of other neuropeptides in this region. The functions they regulate could concern a range of physiological expressions of fear, and it will be interesting to examine whether these can be related, on a cellular level, to the precise output projections that they affect. Such specificity at a cellular level, combined with local differences in neuropeptidergic signalling, may underlie characteristic individualized fear responses. While variations in expression levels of neuropeptide receptors could be one cause, it is equally plausible that differences in release of neuropeptides are at their basis. A better insight into both these mechanisms could be of great importance in view of the current interest in neuropeptide receptors as therapeutic targets for neuropsychiatric disorders.

Abbreviations

BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CeA	central amygdala
CeL	central lateral amygdala
CeM	central Medial amygdala
CRF	corticotropin-releasing hormone
DMN	dorsal motor nucleus of the vagus
MeA	medial amygdala
NPY	neuropeptide Y
NTS	nucleus of solitary tract
PAG	periaqueductal grey
RVLM	rostral ventrolateral medulla
TRH	thyrotropin-releasing hormone

Acknowledgements

The authors wish to thank Drs. Daniel Huber and Fulvio Magara for helpful discussions and Dr. Erwin van den Burg and Mrs. Anouchka Pickenhagen for critical reading of the manuscript.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 19

Multi-factorial somato-dendritic regulation of phasic spike discharge in vasopressin neurons

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Abstract: Classically, neuropeptide release occurs from axon terminals to influence post-synaptic neurons. However, it has become increasingly clear that many neurons in the central nervous system also release neuropeptide from their somata and dendrites. This somato-dendritic neuropeptide release can have many functions, amongst which is feedback modulation of activity. In addition, most central neurons also co-express other neurotransmitters/neuromodulators alongside their principal neurotransmitter, yet the function of these co-expressed factors is largely unknown. With regard to the function of somato-dendritic neuropeptide release, hypothalamic vasopressin neurons are amongst the best understood neurons in the central nervous system. Vasopressin neurons co-express a number of other neuropeptides including apelin, dynorphin and galanin as well as the purine, adenosine triphosphate. In addition to factors co-released during exocytosis, vasopressin neurons also generate nitric oxide. Each of these factors has been demonstrated to influence the activity of vasopressin neurons. For at least some of these factors, modulation of the activity of vasopressin neurons is activity dependent; suggesting that autocrine feedback regulation of activity might be an important role for somato-dendritic release of neuromodulators across the central nervous system.

Keywords: adenosine; afterdepolarization; afterhyperpolarization; apelin; dendrite; dynorphin; galanin; nitric oxide; plateau potential; rhythmic activity

Phasic spike discharge patterning in vasopressin neurons

Secretion of the antidiuretic hormone, vasopressin, is stimulated by increased plasma osmolality or decreased blood volume to return plasma osmolality and blood pressure towards their set-points by promoting antidiuresis and vasoconstriction (Holmes et al., 2001). The somata of vasopressin neurons (and neighbouring oxytocin neurons) are largely found within the hypothalamic paraventricular nucleus and supraoptic nucleus; these neurons project to the posterior pituitary gland and release their peptide products into the general circulation (Leng et al., 1999). Because vasopressin neuron axon terminals do not sustain intrinsic repetitive firing (Bourque, 1990), peripheral vasopressin secretion is largely determined by action potential (spike) discharge initiated at the soma. Under basal conditions, vasopressin neurons display a range of activity patterns, ranging from complete silence, through slow/irregular firing, to

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DOI: 10.1016/S0079-6123(08)00419-6

rhythmic 'phasic' spike discharge (alternating between periods of activity [phasic bursts] and silence that each last tens of seconds) and finally continuous firing. The mechanisms that underpin phasic spike discharge have been extensively studied because this activity pattern appears to enhance the efficiency of stimulus-secretion coupling at the axon terminals in the posterior pituitary gland (Leng et al., 1999).

Phasic firing in vasopressin neurons is largely determined by the intrinsic membrane properties of these neurons, principally non-synaptic post-spike potentials (specifically, afterhyperpolarizations [AHPs] and an afterdepolarization [ADP] (Brown, 2004; Brown and Bourque, 2006)). Nevertheless, synaptic input activity is essential for spike discharge of vasopressin neurons in vivo because antagonism of even a proportion of excitatory synaptic inputs silences vasopressin neurons, even during intense stimulation (Nissen et al., 1994, 1995; Brown et al., 2004a). Bursts are initiated when summated synaptic potentials reach spike threshold. When sufficient spikes occur close enough together, their associated ADPs summate to generate a persistent plateau potential that sustains further firing by bringing the membrane potential closer to threshold for spike initiation (Andrew and Dudek, 1983; Brown et al., 2006). During bursts, a fast AHP appears to enhance spike discharge via activation of a hyperpolarization-activated inward current (I_H) (Ghamari-Langroudi and Bourque, 2000), whilst a medium AHP (mAHP) induces spike frequency adaptation (Kirkpatrick and Bourque, 1996; Greffrath et al., 2004). Activation of a slow AHP (Greffrath et al., 1998; Ghamari-Langroudi and Bourque, 2004) and activity dependent inhibition of ADPs (Brown and Bourque, 2004) decrease plateau potential amplitude, which reduces the probability of spontaneous spikes firing as bursts progress (Brown et al., 2006), eventually resulting in burst termination.

Somato-dendritic neuropeptide release from vasopressin neurons

Vasopressin (and oxytocin) neurons also contain large amounts of neuropeptides within their soma and dendrites (Fig. 1), from which release occurs by exocytosis (Pow and Morris, 1989; Ludwig and Pittman, 2003). This somato-dendritic neuropeptide release is activity dependent (de Kock et al., 2003; Brown et al., 2004b), although it is unlikely that there is tight coupling between individual spikes and individual exocytotic events in the somata/dendrites (Brown et al., 2007). Nevertheless, somato-dendritic exocytosis appears to be critical for phasic spike discharge in vasopressin neurons (Brown, 2004; Brown and Bourque, 2006). The neurosecretory vesicles of vasopressin neurons express V1a and V1b vasopressin receptors (Hurbin et al., 2002), which will presumably be inserted into the cell membrane during exocytosis. Hence activity-dependent vasopressin release will be coupled with activity dependent insertion of surface receptors, providing a mechanism for autocrine activity-dependent feedback by somato-dendritically released vasopressin.

In addition to autocrine and paracrine regulation of spike discharge, somato-dendritic vasopressin release regulates behaviour (e.g. social behaviour and anxiety) by acting as a chemical messenger within the brain (Engelmann et al., 2004; Landgraf and Neumann, 2004; Ludwig and Leng, 2006).

Autocrine modulation of phasic spike discharge by somato-dendritic vasopressin release

Vasopressin is released into the supraoptic nucleus in measurable quantities under basal conditions (Ludwig and Pittman, 2003) and vasopressin administration excites irregular or weakly phasic vasopressin neurons (Gouzenes et al., 1998) but inhibits vasopressin neurons displaying robust phasic spike discharge or continuous spike discharge (Ludwig and Leng, 1997; Gouzenes et al., 1998). Hence, it has been proposed that somato-dendritic vasopressin functions as a 'population feedback signal' that equalizes the average activity level amongst vasopressin neurons (Moos et al., 1998).

The inhibitory actions of vasopressin are probably mediated via modulation of synaptic input strength; vasopressin reduces the amplitude of excitatory postsynaptic currents (EPSCs) (Kombian

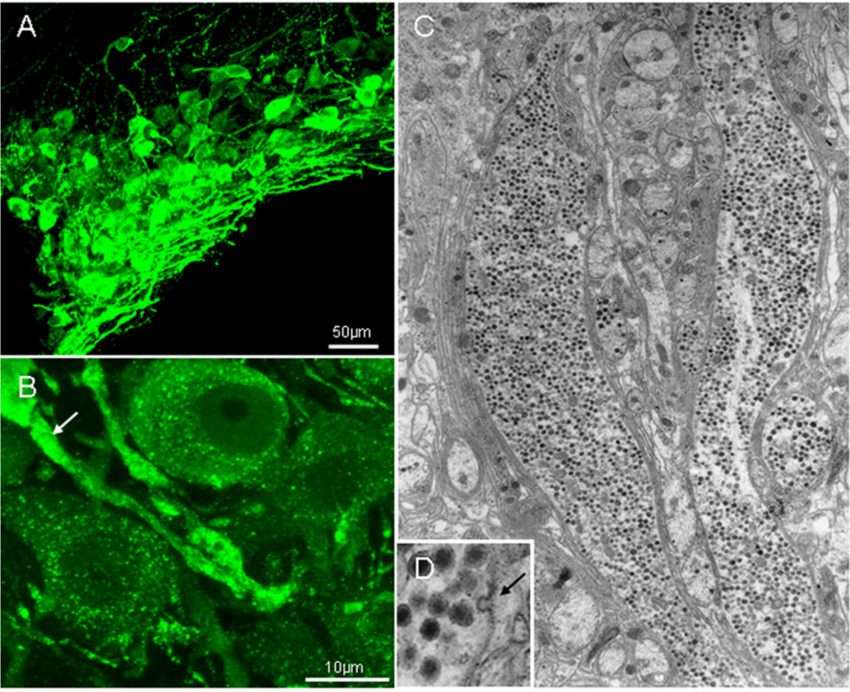

Fig. 1. Expression of vasopressin in the supraoptic nucleus. (A, B) show immunostaining for vasopressin in a coronal section of the rat supraoptic nucleus. (A) In the supraoptic nucleus the dendrites project towards the ventral surface of the brain where they form a dense plexus. (B) The vasopressin immunostaining is punctate and represents individual or aggregates of dense-cored vesicles. In the dendrite thickenings (arrow) the vesicles are particularly abundant giving a very bright signal. (C) The large dense-cored vesicles in the electron-microscopic section of dendrites appear as dark round, membrane-bound organelles. (D) An 'omega' fusion profile at the plasma membrane may show the pit in the dendritic membrane that remains after exocytosis of a dense-cored vesicle (arrow). (See Color Plate 19.1 in color plate section.)

et al., 2000), increases inhibitory postsynaptic current (IPSC) frequency (Hermes et al., 2000), but does not affect post-spike potentials (Brown and Bourque, 2004). The mechanism of vasopressin excitation of vasopressin neurons is less clear. However, vasopressin stimulates adrenocorticotropic hormone secretion from anterior pituitary corticotrophs, via V1bR coupled to phospholipase C (PLC) (Volpi et al., 2004) and vasopressin also activates PLC in vasopressin neurons. Hence, V1bR mediated activation of PLC might underpin the vasopressin induced excitation of spike discharge in vasopressin neurons (Sabatier et al., 1998).

Whilst exogenous vasopressin can excite or inhibit spike discharge in vasopressin neurons, to date only inhibitory actions of endogenous vasopressin have been identified; antagonism of supraoptic nucleus V1aRs prolongs phasic bursts and increases spike discharge by $\sim$1 Hz during bursts in vivo (Ludwig and Leng, 1997; Brown et al., 2004b). Hence, it appears that vasopressin neurons are continuously exposed to endogenous vasopressin at levels that reflect the average activity of the vasopressin neuron population as whole. Indeed, V1aR antagonist induced increase in firing rate is constant throughout bursts, indicating that endogenous vasopressin tonically inhibits phasic vasopressin neurons (Fig. 2).

Autocrine modulation of phasic spike discharge by co-released neuropeptides

In addition to vasopressin itself, vasopressin neurons synthesize and secrete several other neuropeptides that have been implicated in modulation of phasic spike discharge in vasopressin neurons,

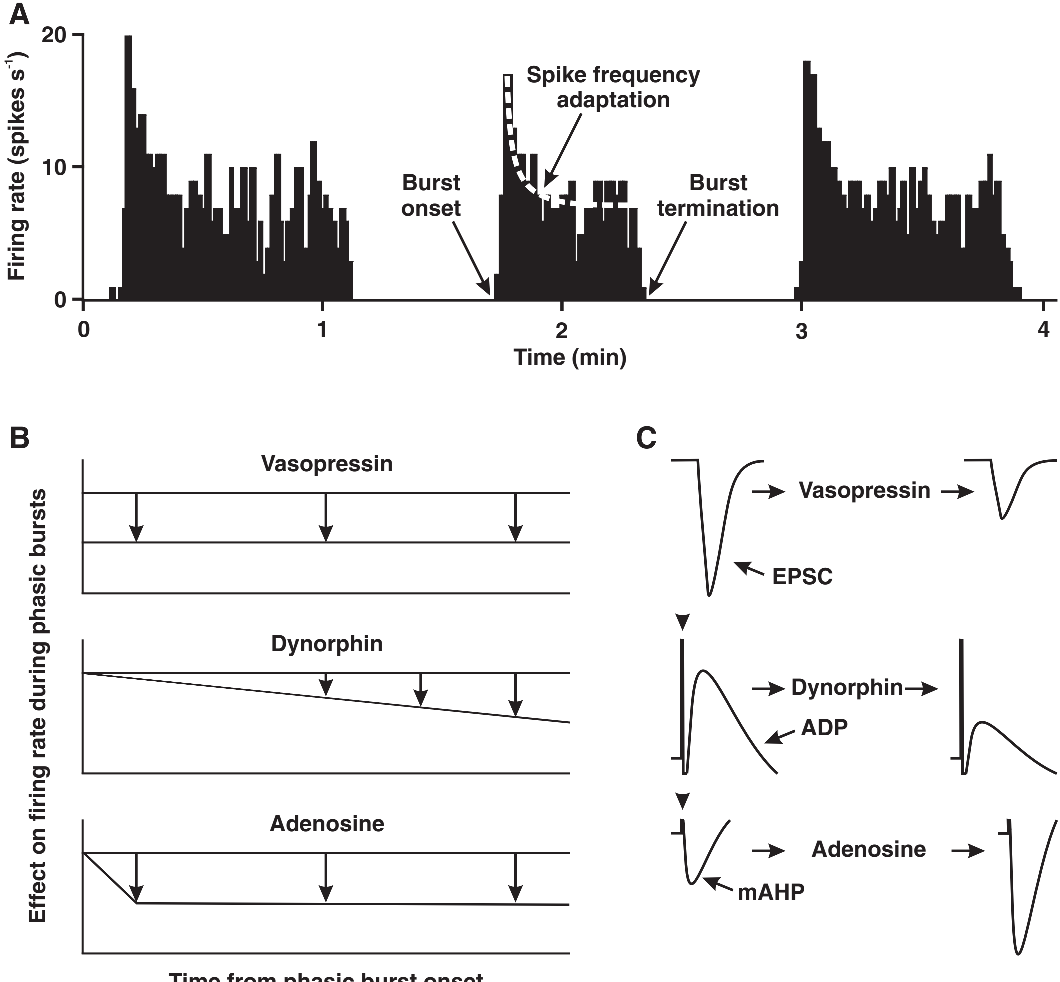

Fig. 2. Mechanisms of somato-dendritic regulation of phasic spike discharge in vasopressin neurons. (A) Ratemeter recording of the electrical activity of a vasopressin neuron from the supraoptic nucleus of a urethane-anaesthetized rat, exhibiting spontaneous phasic spike discharge. Note the clear periods of activity and silence that each last several tens of seconds and that each start and stop abruptly. Note also the clear spike frequency adaptation that occurs over the course of each burst (dashed white line). (B) Schematic representation of the temporal effects of some autocrine modulators of phasic spike discharge of vasopressin neurons (not to scale): endogenous vasopressin inhibits spike discharge throughout bursts (Brown et al., 2004b), endogenous dynorphin inhibition increases progressively over the course of bursts (Brown et al., 2004b) and endogenous adenosine inhibition increases over the first few seconds of bursts and remains relatively constant thereafter (Bull et al., 2006). (C) The major actions of autocrine modulators that appear to be responsible for their temporal effects on phasic spike discharge (not to scale): vasopressin inhibits EPSC amplitude (Kombian et al., 2000), dynorphin inhibits ADP amplitude (Brown and Bourque, 2004) and adenosine enhances mAHP amplitude (Ruan and Brown, unpublished observations).

including dynorphin (Watson et al., 1982), galanin (Landry et al., 2003) and apelin (De Mota et al., 2004). Vasopressin neurons express receptors for each of these peptides (O'Donnell et al., 1999; Shuster et al., 1999; Burazin et al., 2001; O'Carroll and Lolait, 2003), providing a mechanism for each to modulate vasopressin neuron spike discharge.

Dynorphin

The κ-opioid peptide, dynorphin, and κ-opioid receptors are co-localized within vasopressin neuron neurosecretory vesicles (Whitnall et al., 1983; Shuster et al., 1999) and endogenous activation of supraoptic nucleus κ-opioid receptors is essential

for the expression of phasic spike discharge by vasopressin neurons in vivo; chronic administration of a κ-opioid agonist into supraoptic nucleus desensitizes κ-opioid receptors and eliminates phasic spike discharge in vasopressin neurons, even during osmotic stimulation (Brown et al., 1998).

Similar to V1aR antagonism, acute administration of a κ-opioid receptor antagonist into the supraoptic nucleus increases spike discharge by ~1 Hz during phasic bursts in vivo and prolongs phasic bursts in vivo (Brown et al., 1998) and in vitro (Brown and Bourque, 2004), consistent with somato-dendritic release of endogenous dynorphin to inhibit phasic spike discharge.

However, the mechanism of the κ-opioid inhibition of spike discharge in phasic neurons is different from that of the vasopressin inhibition. Whilst κ-opioid receptor agonists inhibit synaptic inputs (Inenaga et al., 1994), they also inhibit the ADP (Brown et al., 1999) and it is the latter effect that appears to be of importance in the κ-opioid modulation of phasic spike discharge (Brown and Bourque, 2006); activity-dependent inhibition of the ADP is caused by activity-dependent activation of κ-opioid (but not μ-opioid) receptor by endogenous κ-opioids (Brown and Bourque, 2004) to decrease plateau potential amplitude during phasic bursts (Brown et al., 2006).

Another important difference between the endogenous vasopressin and κ-opioid inhibition of phasic spike discharge is that κ-opioid inhibition is absent at the onset of each burst but increases over the course of each burst (Brown et al., 2004b). Hence, the κ-opioid inhibition is itself activity dependent, becoming progressively more powerful during bursts and so might be critical for phasic spike discharge in individual neurons (Fig. 2).

Galanin

Like dynorphin, galanin is co-expressed in vasopressin neurosecretory granules. However, some neurosecretory granules contain galanin, but not vasopressin, and this population is the most abundant in the dendrites of vasopressin neurons (Landry et al., 2003). Thus, galanin might be specifically targeted to the dendrites to serve an autocrine/paracrine function.

Galanin effects on peripheral vasopressin secretion depend on the osmotic status of the animal. In vivo i.c.v. galanin administration increases peripheral vasopressin secretion under basal conditions but inhibits secretion during dehydration (Ciosek and Cisowska, 2003). However, to date only (multiple) inhibitory effects of galanin on supraoptic nucleus neurons have been identified, and so the stimulatory actions of galanin might be mediated via distant actions on afferent inputs that are overridden by the local inhibitory actions during dehydration. Galanin reduces evoked EPSCs in supraoptic nucleus neurons, probably via presynaptic GAL2/3 receptors (Kozoriz et al., 2006). Galanin also directly hyperpolarizes supraoptic nucleus neurons (Papas and Bourque, 1997), an effect that is increased in dehydrated rats (Kozoriz et al., 2006), perhaps as a result of supraoptic nucleus GAL1 receptor up-regulation during dehydration (Burazin et al., 2001). Hence, galanin might provide an important restraint mechanism to prevent over-excitation of vasopressin neurons during dehydration. [Indeed, it has been known for some time that galanin can reduce peripheral vasopressin secretion in response to administration of hypertonic saline (Kondo et al., 1991).]

Perhaps of more importance to phasic spike discharge, galanin also inhibits the ADP to reduce evoked spike discharge in vasopressin neurons (Papas and Bourque, 1997). However, the effects of endogenous galanin on phasic spike discharge have not been investigated in vivo and it is not known whether galanin effects on the ADP are activity dependent, as is the case for dynorphin (Brown and Bourque, 2004). Nevertheless, somato-dendritic release of galanin is likely to be an important modulator of vasopressin neuronal activity, particularly when vasopressin neuron activity is stimulated by dehydration.

Apelin

Apelin, a novel peptide originally isolated from bovine stomach tissue extracts, is widely but selectively distributed throughout the nervous system and the vasopressin neurons of the supraoptic and paraventricular nucleus represent some of the most apelin-rich regions in the brain, with a dense network of apelin-immunoreactive nerve cell bodies,

dendrites and axons (Brailoiu et al., 2002; Reaux et al., 2002). High-resolution confocal microscopic images of magnocellular hypothalamic neurons show a marked segregation of apelin and vasopressin immunoreactivity within supraoptic and paraventricular nuclei neurons, suggesting that the two peptides might be stored in, and therefore differentially released from, two distinct vesicular pools within the same cells (Reaux-Le Goazigo et al., 2004).

Intracereboventricular (i.c.v.) injection of apelin decreases spike discharge in vasopressin neurons of lactating animals (De Mota et al., 2004) and inhibits basal and dehydration-induced vasopressin release (Reaux et al., 2001; De Mota et al., 2004), suggesting an inhibitory role of apelin in the regulation of vasopressin release. Because vasopressin neurons synthesize apelin (De Mota et al., 2004) and the apelin receptor is expressed by vasopressin neurons (O'Carroll and Lolait, 2003), it has been suggested that apelin acts as an autocrine feedback inhibitor of vasopressin neuron activity and that a reduction in apelin inhibition during dehydration might facilitate phasic spike discharge of vasopressin neurons (De Mota et al., 2004). By contrast to the effects of i.c.v. apelin, we have recently found that administration of apelin directly into the supraoptic nucleus increases vasopressin neuron activity and that it might do so (at least in part) by inhibiting somato-dendritic vasopressin release. As mentioned above, dendritically-released vasopressin has a predominantly inhibitory action on vasopressin cells and thus, reducing somato-dendritic vasopressin release by apelin might explain the increase in vasopressin cell activity (Ludwig et al., 2005).

Autocrine modulation of phasic spike discharge by other factors

Adenosine

In addition to activity-dependent release of adenosine and adenosine triphosphate (ATP) from glial cells or synaptic inputs, the magnocellular neurons themselves (via a combination of adenosine secretion and the rapid catabolism of exocytosed ATP (Song and Sladek, 2005)) contribute to the extracellular adenosine concentration. Similar to other neurons, vasopressin neurosecretory granules contain ATP (Poisner and Douglas, 1968), which is presumably co-released upon somato-dendritic exocytosis of neuropeptides. Although ATP induces vasopressin secretion from hypothalamic explants, these effects are truncated by rapid catabolism to adenosine in the extracellular space (Kapoor and Sladek, 2000); this adenosine also influences the activity of vasopressin neurons.

As in other brain areas, the major effects of adenosine on vasopressin neurons are mediated via A1 and A2A receptors (Oliet and Poulain, 1999; Noguchi and Yamashita, 2000; Ponzio and Hatton, 2005; Bull et al., 2006; Ponzio et al., 2006). In anesthetized rats, A1 receptor activation inhibits vasopressin neurons (Bull et al., 2006). Several potential mechanisms that might mediate this inhibition have been identified in supraoptic neurons in vitro: reduction of voltage-dependent calcium currents (Noguchi and Yamashita, 2000), inhibition of glutamate (and GABA) release via pre-synaptic receptors, reduction of action potential duration and hyperpolarization through postsynaptic receptors (Oliet and Poulain, 1999; Ponzio and Hatton, 2005). A2A receptors are expressed by supraoptic neurons (and glia) and activation of these receptors induces depolarization of supraoptic neurons to increase spike discharge (Ponzio et al., 2006). Nevertheless, the major functional role of endogenous adenosine appears to be inhibition because an adenosine uptake inhibitor strongly inhibits spike discharge (Ponzio and Hatton, 2005).

The major effects of endogenous adenosine on phasic spike discharge in vivo are activity dependent: enhancement of spike frequency adaptation and reduction of burst duration (Bull et al., 2006) (Fig. 2). Spike frequency adaptation is induced by activation of the mAHP in vitro (Kirkpatrick and Bourque, 1996) and we now have preliminary evidence to suggest that endogenous adenosine enhances the mAHP to reduce phasic burst duration and decrease firing rate during bursts (Ruan and Brown, unpublished observations).

Whilst the effects of endogenous adenosine on phasic spike discharge are dependent on the activity of the individual neuron, it is possible that

the source of adenosine that affects vasopressin neurons is not ATP released by the vasopressin neurons themselves; the amplitude of miniature EPSCs is increased by glial ATP release in the paraventricular nucleus (Gordon et al., 2005).

Nitric oxide

Vasopressin neurons express nitric oxide synthase (Hatakeyama et al., 1996) and selective inhibition of neuronal nitric oxide synthase increases peripheral secretion of vasopressin in response to an osmotic stimulus (Ventura et al., 2005), suggesting that endogenous nitric oxide production restrains peripheral vasopressin secretion. By contrast, nitric oxide production promotes osmotically induced somato-dendritic vasopressin release because blockade of nitric oxide production (or addition of nitric oxide scavengers) reduces somato-dendritic vasopressin release (Gillard et al., 2007). Hence the observed effects of nitric oxide on peripheral vasopressin secretion might be mediated by its effects on somato-dendritic secretion.

Whether its effects are mediated by modulation of somato-dendritic vasopressin release, nitric oxide inhibits spike discharge of supraoptic nucleus neurons in vitro (Liu et al., 1997; Stern and Ludwig, 2001) and in vivo (Stern and Ludwig, 2001) by increasing the frequency (and amplitude) of IPSCs in vasopressin neurons (Ozaki et al., 2000; Stern and Ludwig, 2001), as does vasopressin itself (Hermes et al., 2000). However, inhibition by nitric oxide production is not limited to neurons displaying phasic spike discharge; nitric oxide also inhibits continuously active vasopressin (and oxytocin neurons) (Stern and Ludwig, 2001).

Conclusion

It is clear that several autocrine mechanisms (of which we have highlighted only a select few) serve essentially the same function; to restrain the activity of vasopressin neurons, particularly at times when secretion is stimulated. The question remains as to why so many different mechanisms do so? The simple answer might be that prevention of vasopressin over-secretion is so important for the survival of the organism that multiple redundancies are built in to the system; failure in any one (or more) system(s) can then be compensated by the remaining systems. Whether this is indeed the case has yet to be determined.

Additionally, somato-dendritically released factors might (like vasopressin itself) act as chemical messengers within the brain that modulate the activity of other brain systems; e.g. apelin stimulates the hypothalamo-pituitary-adrenal axis by activation of corticotrophin releasing hormone neurons localized alongside vasopressin neurons in the paraventricular nucleus (Jaszberenyi et al., 2004).

At least some of these autocrine feedback systems appear to do more than simply restrain activity. For several systems there is good evidence that autocrine feedback drives vasopressin neurons towards adopting a phasic spike discharge pattern (which is most efficient for peripheral vasopressin secretion): endogenous κ-opioid receptor activation inhibits the ADP (Brown and Bourque, 2004) to contribute to burst termination (Brown et al., 1998) and endogenous A1 receptor activation enhances the mAHP (Ruan and Brown, unpublished observations) to increase spike frequency adaptation at the onset of bursts (Bull et al., 2006). Other autocrine factors target synaptic inputs and/or intrinsic membrane properties and modulate phasic spike discharge: vasopressin reduces EPSC amplitude (Kombian et al., 2000) and increases IPSC frequency (Hermes et al., 2000); galanin reduces ADP (Papas and Bourque, 1997) and EPSC amplitude (Kozoriz et al., 2006); nitric oxide increases IPSC frequency (Ozaki et al., 2000; Stern and Ludwig, 2001). To date, the mechanism of apelin actions on vasopressin neurons is unknown but (similarly to nitric oxide) might involve modulation of somato-dendritic vasopressin release. Hence, in addition to providing for multiple redundancy of autocrine feedback inhibition of vasopressin neurons spike discharge, the various neurotransmitter/neuromodulators released from the soma and dendrites of these neurons might contribute to different aspects of phasic spike patterning to optimize the efficiency of stimulus–secretion coupling at the axon terminals.

Abbreviations

ADP	afterdepolarization
AHP	afterhyperpolarization
ATP	adenosine triphosphate
EPSC	excitatory postsynaptic current
GABA	γ-aminobutyric acid
IPSC	inhibitory postsynaptic current
mAHP	medium afterhyperpolarization
PLC	phospholipase C
V1aR	vasopressin V1a receptor
V1bR	vasopressin V1b receptor

Acknowledgements

This work was supported by grants from the BBSRC (Mike Ludwig), the Wellcome Trust (Colin H. Brown) and the Lottery Grants Board of New Zealand (Colin H. Brown).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 20

Neurophysiology of supraoptic neurons in C57/BL mice studied in three acute in vitro preparations

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Abstract: Osmotic control of arginine vasopressin (AVP) and oxytocin (OXT) release from magnocellular neurosecretory cells (MNCs) of the supraoptic (SON) and paraventricular (PVN) nuclei is essential for body fluid homeostasis. The electrical activity of MNCs, which is regulated by intrinsic and extrinsic osmosensitive factors, is a primary determinant of blood AVP and OXT levels. Although we now understand many of the cellular mechanisms that mediate the osmotic control of electrical activity and secretion from MNCs, further insight is likely to emerge from a molecular analysis of these mechanisms. An important step towards this goal could be made through the use of mouse genetic models. However, the electrophysiological properties of MNCs in mice have not been characterized, making direct comparisons with the rat model somewhat difficult. In this study, we examined the electrical properties of MNCs from the mouse SON. Extracellular recordings from neurons in superfused explants revealed modes of basal and osmotically modulated firing very similar to those observed previously in rats. Recordings in hypothalamic slices confirmed that SON neurons receive kynurenic-acid-sensitive excitatory synaptic inputs from the organum vasculosum laminae terminalis (OVLT). Current-clamp recordings from acutely dissociated SON neurons showed proportional changes in membrane cation conductance during changes in fluid osmolality. We conclude, therefore, that MNCs in the mouse SON display intrinsic osmosensitive properties and firing patterns that are very similar to those reported in the rat. Mouse MNCs therefore represent a useful model for the study of molecular factors contributing to the osmotic control of AVP and OXT release.

Keywords: electrical activity; firing pattern; osmosensitivity; supraoptic nucleus; mouse; hypothalamic slice; hypothalamic explant; immunocytochemistry

Introduction

Activity of supraoptic neurons and neurohypophysial secretion

Arginine vasopressin (AVP) and oxytocin (OXT) are peptide hormones synthesized in the somata of separate populations of magnocellular neurosecretory cells (MNCs) in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. These cells project their axons into the neurohypophysis, where hormone release is controlled primarily by calcium-dependent exocytosis triggered by the arrival of action potentials initiated at MNC somata (Poulain and Wakerley, 1982; Bourque and Renaud, 1990). Although the electrical activity of AVP-containing MNCs and

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DOI: 10.1016/S0079-6123(08)00420-2

corresponding release of AVP can be modulated by a variety of stimuli to affect various peripheral organs (Cunningham and Sawchenko, 1991), the peptide is best known for its role as the body's antidiuretic hormone and for its role in systemic osmoregulation (Dunn et al., 1973; Robertson et al., 1976). In rats, hyperosmolality has been shown to enhance action potential firing rate and to promote phasic bursting activity from AVP-releasing MNCs to stimulate AVP release and water reabsorption (Walters and Hatton, 1974; Wakerley et al., 1978). Conversely, decreases in plasma osmolality inhibit the electrical activity of MNCs and AVP secretion from the neurohypophysis, thereby promoting water excretion (Brimble and Dyball, 1977).

Mechanisms contributing to the osmotic control of AVP MNCs

Previous studies in rats have indicated that a variety of mechanisms contribute to the osmotic control of electrical activity in MNCs during systemic osmotic perturbations (Hussy et al., 2000; Voisin and Bourque, 2002). For example, MNCs are depolarized by hypertonic stimuli and hyperpolarized by hypotonicity because they express stretch-inhibited cation channels whose activity varies inversely with cell volume (Oliet and Bourque, 1993a, b). These effects are assisted by surrounding glial cells which release taurine as an inverse function of osmolality (Deleuze et al., 1998). Taurine is a potent agonist at extra-synaptic, strychnine-sensitive, glycine receptors on MNCs (Hussy et al., 1997; Deleuze et al., 2005) and experiments in vivo have shown that enhanced taurine release during hypotonicity inhibits firing, whereas reduced taurine release and deactivation of glycine receptors can excite MNCs under hypertonic conditions (Hussy et al., 1997). Patch-clamp studies have also shown that MNCs express stretch-activated K^+ channels (Han et al., 2003) whose activity might be affected by volume changes that result from osmotic stimulation (Zhang and Bourque, 2003) and thus contribute to the osmotic control of membrane potential. A recent study has shown also that acute hypertonicity enhances a slow voltage-activated K^+ current that might participate in the control of firing pattern of MNCs (Liu et al., 2005).

Osmotic stimuli may also regulate MNCs via osmosensitive neurons located in other parts of the brain and periphery that send axons to the SON and PVN (Bourque et al., 1994). Notably, experiments in vivo have shown that osmosensitive elements in the region of the antero-ventral third ventricle can regulate the firing of MNCs in both the PVN (Honda et al., 1987) and SON (Honda et al., 1990). The modulation of MNCs in the SON by osmosensitive neurons in the organum vasculosum laminae terminalis (OVLT) has been shown to be mediated specifically by excitatory synapses involving ionotropic glutamate receptors (Richard and Bourque, 1995).

Even more complex forms of regulation also take place during protracted perturbations. For instance, chronic hyperosmotic and hypoosmotic stimuli are known to modulate gene expression in MNCs (Glasgow et al., 2000; Hindmarch et al., 2006), and to affect the density of Na^+ channels (Tanaka et al., 1999), Ca^{2+} channels (Zhang et al., 2007) and NMDA receptors (Curras-Collazo and Dao, 1999). Finally, chronic hyperosmolality has been shown to modify neuro-glial interactions within the SON and PVN (Miyata and Hatton, 2002; Theodosis et al., 2004), where it might significantly impact the taurine-mediated and synaptic control of MNCs (Oliet et al., 2004).

The mouse as a model system for studying the osmotic regulation of AVP MNCs

A more detailed and mechanistic understanding of the osmotic control of MNCs now requires that these processes be investigated at the molecular level. Studies in other physiological systems have shown that the use of murine genetic models provides a powerful means to probe the involvement of specific genes in a variety of cellular and molecular processes (Takahashi et al., 1994; Nelson, 1997). Although osmoregulated AVP release has been studied in mice (Morris et al., 1999; Chen et al., 2005; Sharif-Naeini et al., 2006), very little is known concerning the basic osmotic neurophysiology of MNCs in these animals. Here we used immunocytochemistry and a variety of

electrophysiological approaches to investigate basic neurophysiological features of mouse MNCs in three different acute in vitro preparations of SON: isolated cells, superfused explants and submerged horizontal slices. Our results reveal differences in the density of OXT-and AVP-releasing MNCs in the SON of mice compared to rats, but they also indicate that the basic firing patterns and osmoresponsive properties of these cells are quite similar to those that have been reported in rats.

Methods

Animals

Six to eight weeks old male C57/BL mice (Charles River Laboratories, Inc., Québec, Canada) were used in this study. Animals were treated in strict accordance with the guidelines outlined by the Canadian Council on Animal Care (http://www.ccac.ca) and to protocols approved by the Animal Care Committee of McGill University. For all electrophysiological and immunocytochemical experiments, mice were deeply anaesthetized using halothane (Sigma-Aldrich Co.), decapitated and the tissue was prepared as described in the sections below.

Immunohistochemistry on tissue sections

Mice were perfused transcardially with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and serial coronal sections (30 μm thick) were cut through the hypothalamus. Sections were blocked for 1 h at room temperature in 4% normal goat serum (NGS), 0.5% bovine serum albumin, 0.1% Triton-X followed by incubation with a rabbit anti-rat-OXT (VA-10 antibody; 1/1000; Altstein and Gainer, 1988) and mouse anti-AVP-neurophysin antibody (PS41; 1/200; Ben-Barak et al., 1985, both gifts from Dr. H. Gainer) overnight at 4°C. Secondary antibodies (Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit, both diluted 1:500 in PBS; Invitrogen Canada, Inc., Burlington, ON) were applied for 30 min. Fluorescence images were acquired using a spinning-disc confocal microscope equipped with an ORCA-ER camera and MetaMorph Imaging software (PerkinElmer Biosignal, Inc., Montreal, Québec).

Immunocytochemistry on isolated MNCs

Small blocks of tissue ($<1\,mm^3$) containing the SON were excised using small scissors and incubated for 30 min at room temperature in oxygenated PIPES solution containing (in mM): NaCl (130), KCl (5), $MgCl_2$ (1), PIPES (20), $CaCl_2$ (1), glucose (10), as well as $0.5\,mg\,ml^{-1}$ protease X and $0.5\,mg\,ml^{-1}$ protease XIV (Sigma-Aldrich Co.). All solutions were adjusted to $312\,mosmol\,kg^{-1}$ using mannitol, close to the average value of serum osmolality in this strain of mice (Ciura and Bourque, 2006; Sharif-Naeini et al., 2006). SONs were then transferred to a protease-free PIPES solution, triturated and plated onto treated cell culture dishes (No. 430165 Corning, Inc.) for 30 min before being fixed overnight in 4% PFA in PBS. The dishes were then washed in PBS, incubated at room temperature for 1 h in PBS, 1% NGS, 0.3% Triton X-100, then incubated with primary antibodies overnight at 4°C. The primary antibodies were PS 38 mouse anti-OXT-neurophysin (1/200), and PS 41 mouse anti-AVP-neurophysin (1/200) (generously provided by Dr. Hal Gainer). Dishes were then washed in PBS and incubated in biotinylated goat anti-mouse secondary (1/500, AP124B Chemicon International, Inc.) for 2 h, then washed in PBS. Detection of immunolabelling was obtained by incubating in Avidin–Biotin Complex reagent (PK-4000, Vector Laboratories Ltd., Burlingame, CA) for 30 min and exposing to diaminobenzidine substrate (SK-4100, Vector Laboratories Ltd.) for 3–4 min. The short (R_s) and long (R_l) radii of phase bright cells were measured using a calibrated reticule and the cross-sectional area (CSA, in μm^2) of each cell was estimated as that of a simple oval (i.e. $CSA = \pi R_s R_l$).

Extracellular recordings from acute hypothalamic explants

For electrophysiological recordings in hypothalamic explants, the brain was removed and a block of tissue ($7 \times 7 \times 2\,mm$) comprising the basal hypothalamus was excised using razor blades and

pinned, ventral surface up, to the Sylgard® base of a temperature-controlled (32–33°C) chamber. Explants were superfused (1.5 ml min^{-1}) with an oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (ACSF, pH 7.4) composed of (in mM): NaCl (125), $NaHCO_3$ (26), KCl (5), $MgCl_2$ (1.5), $CaCl_2$ (1), D-glucose (10), delivered via a Tygon® tube placed over the caudal end of the optic tract. Mannitol was added to adjust the basal osmolality of the solution to 312 mosmol kg^{-1}, or to make hypertonic solutions. Extracellular recordings were made from SON neurons using microelectrodes filled with 1 M potassium acetate (5–10 MΩ) and an Axoclamp 2A amplifier (Molecular Devices Corp., Sunnyvale, CA). The voltage output of the amplifier was filtered (0.6–1.5 kHz) and amplified 100 times prior to digital capture using a Digidata 1200B interface and pCLAMP 8 software (Molecular Devices Corp.). Spikes were detected off-line to construct plots of firing rate as a function of time. During these recordings, osmotic stimuli were bath-applied by switching the solution being delivered through the Tygon® tube.

Patch-clamp recordings from angled horizontal hypothalamic slices

Freshly isolated brain was rapidly transferred cortex side down into a dish containing cold (0–4 °C) oxygenated (95% O_2; 5% CO_2) ACSF composed of (in mM): NaCl (124), KCl (4.96), NaH_2PO_4 (1.23), $MgCl_2$ (1.48), $CaCl_2$ (2.52), $NaHCO_3$ (26) and D-glucose (10). The brain was trimmed in the coronal and sagittal planes to isolate the hypothalamus, and this block was then glued (with the rostral end high) to a mounting stage with an inclination angle of 42° (Trudel and Bourque, 2003). A single 400 μm thick slice was cut in cold ACSF using a vibratome and this slice was then placed dorsal side up in a recording chamber mounted on a fixed-stage upright microscope (BX51, Olympus America Ltd.). The slice was held in place by tight nylon threads attached to a C-shaped platinum rod (approximately 2 cm diameter), and perfused at 2–3 ml min^{-1} with oxygenated ACSF warmed to 32–34°C. Whole-cell patch-clamp recordings (2–3 h after preparation) were performed using microelectrodes (2–4 MΩ) filled with (in mM): K-Gluconate (140), $MgCl_2$ (2), HEPES (10), Na_2ATP (2) and Na-GTP (0.4). Whole-cell current and voltage were recorded using an Axoclamp 1D amplifier and digitized as explained earlier. Electrical stimulation was triggered by the acquisition system and delivered by an isolated stimulator through a pair of Teflon-coated platinum-iridium wires. The coating was removed at the tip of the electrodes and one tip was inserted in the tissue, while the other was left in the ACSF. Bicuculline was dissolved directly in the ACSF at a concentration of 5 μM. Kynurenic acid (KYN) was first dissolved in a small quantity of 0.5N NaOH before being dissolved in ACSF at a final concentration of 5 mM.

Experiments on isolated MNCs

Dishes containing live, acutely isolated cells obtained as explained above (<2 h after plating) were placed on the stage of an inverted phase-contrast microscope (Nikon Ltd.) and perfused with a HEPES-buffered saline solution (pH 7.3) containing (in mM): NaCl (130), KCl (3), $MgCl_2$ (1), HEPES (10), $CaCl_2$ (1), glucose (10), adjusted to 312 mosmol kg^{-1} with mannitol. Perfusion at 0.2 ml min^{-1} was achieved via a three-barrel assembly controlled by a fast stepper device (Warner Instruments Co., Hamden, CT), allowing rapid (<20 ms) switching between solutions. Hypertonic (372 mosmol kg^{-1}), or hypotonic (272 mosmol kg^{-1}) stimuli were applied via HEPES solution containing more or less mannitol, respectively. Cells were patch-clamped with microelectrodes (4–7 MΩ) containing a solution (pH 7.34) comprising (in mM): K-Gluconate (120), $MgCl_2$ (1), EGTA (1), HEPES (10), ATP (4), GTP (1), phosphocreatine (14), adjusted to 280 mosmol kg^{-1} with mannitol. Recordings were done in the whole-cell mode using an Axopatch-200B amplifier (Molecular Devices Corp.) and membrane current (d.c.−2 kHz) was digitized as explained earlier. Whole-cell capacitance and series resistance were neutralized electronically. In current-clamp experiments, osmotic stimuli were applied for 120 s, and the membrane potential (excluding action potentials and afterhyperpolarisations) and firing frequency were measured during a 10 s interval before the stimulus (baseline) and before the end of the stimulus (osmotic response). Where required,

membrane input resistance (R_i) was calculated from the steady state voltage response (ΔV) to a hyperpolarizing current pulse (ΔI) as $R_i = \Delta V/\Delta I$.

Measurement of changes in cell volume

Relative changes in cell volume (ΔVol, in %) were determined from values of maximal CSA as previously described (Zhang and Bourque, 2003). For each image, the perimeter of the cell was traced using Scion Image for Windows 4.02 (Scion Corp., Frederick, MA) and the CSA (in pixels) was determined. Values of CSA measured during control were averaged (CSA_0) and values of ΔVol at different time points (ΔVol_t) were calculated from the corresponding value of CSA (CSA_t) using the equation: $\Delta Vol_t = 100 \times [(CSA_0^{1.5} - CSA_t^{1.5})/CSA_0^{1.5}]$.

Statistics

All values in this paper are reported as mean plus or minus the standard error of the mean (±SEM). Comparisons of the means between groups were made using a paired *t*-test or a two-way analysis of variance (ANOVA, SigmaStat 2.1, SPSS Science, Inc., Chicago, IL), as appropriate. Differences between the means were considered significant when $p<0.05$. Where differences were found, the Student–Newman–Keuls test for multiple comparisons was performed post hoc to identify specific distinctions ($p<0.05$). In comparing input resistances, a one-way repeated measures ANOVA was used with Tukey's post hoc test.

Results

AVP and OXT neuron distribution in mouse SON

To examine the relative distribution of AVP and OXT neurons in the C57/BL mouse SON, we performed immunohistochemical staining on coronal sections (taken 90 μm apart) using specific antibodies (see "Methods"). The SON was found to be a well-delimited hypothalamic nucleus lying immediately lateral to the optic tract along the ventral surface of the brain. The rostral pole of the nucleus was located just behind the coronal plane defined by the junction between the optic nerves and the optic chiasma, and extended approximately 350–400 μm in the caudal direction from this point. As illustrated in Fig. 1, the shape of the nucleus was wide (~250 μm) and shallow (~40 μm) at its rostral pole and gradually changed proportions to appear tall (~125 μm) and narrow (~65 μm) at the caudal end. Overall, the mouse SON was densely populated with the somata of MNCs. A majority of these neurons were found to contain AVP. Indeed, an analysis of 20 sections taken at intervals of 90 μm through each of 4 SONs revealed that AVP neurons account on average for 77.4±1.6% of the MNCs present in this nucleus. The more sparse OXT-positive neurons were generally found to be present in the dorsal and rostral aspects of the nucleus (Fig. 1).

Spontaneous activity patterns of mouse SON neurons in acute explants

To characterize the basal activity patterns of mouse MNCs, we obtained extracellular single unit recordings from 56 SON neurons in acute explants of mouse hypothalamus superfused with ACSF adjusted to an osmolality of 312 mosmol kg^{-1}, a value corresponding to the basal serum osmolality of C57/BL mice in vivo (Sharif-Naeini et al., 2006). Although these neurons were not specifically identified as AVP- or OXT-containing, the cells recorded were mainly located in the caudo-ventral part of the nucleus and were therefore most likely AVP MNCs. As illustrated in Fig. 2A, the action potential firing modes displayed by spontaneously active SON neurons could be divided into three main categories. *Slow irregular firing*, arbitrarily defined as those neurons displaying an average rate of action potential discharge less than 2 Hz and with a high incidence of silent pauses lasting >1 s, was observed in 25% ($n = 14$) of the cells recorded. *Continuous firing*, characterized by a steady rate of firing at frequencies ≥2 Hz, was observed in 59% ($n = 33$) of the recorded cells. Finally, *phasic firing*, a mode of activity characterized by consecutive periods of silence and steady firing (3–12 Hz) lasting tens of seconds each, was observed in 16% ($n = 9$) of the cells recorded under resting conditions.

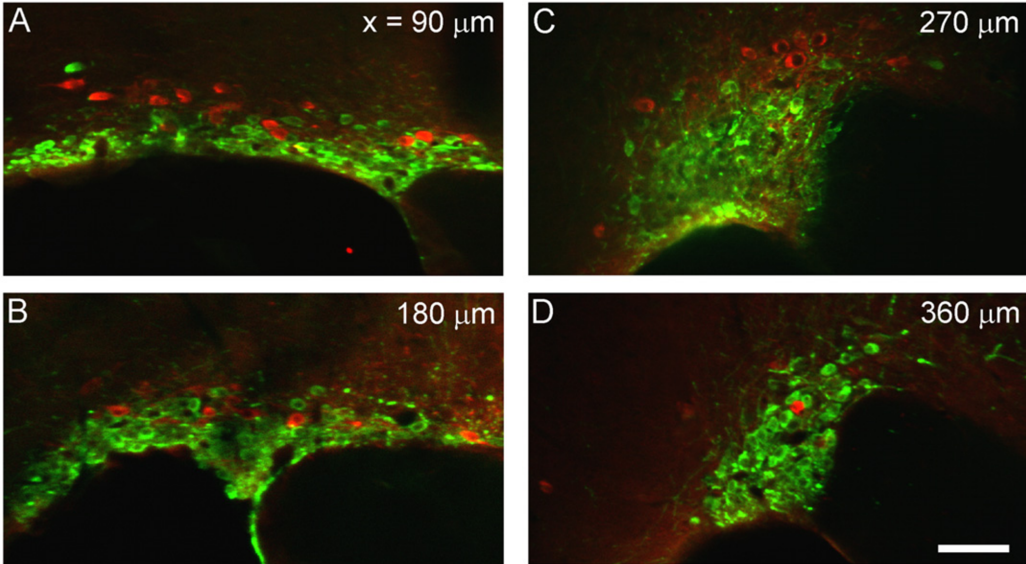

Fig. 1. Distribution of MNCs in the SON of C57/BL mice. Fluorescence micrographs showing AVP (green) and OXT (red) neurons in the SON of a C57/BL mouse. Panels show images taken from coronal sections taken 90 μm (A), 180 μm (B), 270 μm (C) and 360 μm (D) from the rostral pole of the nucleus. Note that OXT neurons are scattered in the dorsal part of the nucleus which is otherwise dominated by AVP neurons. Scale bar = 50 μm. (See Color Plate 20.1 in color plate section.)

Osmosensitivity of mouse SON neurons in acute explants

We next examined the effects of hypertonic and hypotonic stimuli on SON neurons in superfused hypothalamic explants. Although cells displaying phasic firing appeared to be excited by hypertonicity and inhibited by hypotonicity, the number of cells tested was insufficient to characterize these responses statistically. We therefore analyzed the effects of osmotic stimulation on the mean overall firing rate of cells showing slow irregular or continuous firing, and we restricted this analysis to cells that did not develop phasic firing during the stimulus. Bath application of a hyperosmotic solution (+25 mosmol kg^{-1}) was found to provoke a significant increase in the firing rate of 24 cells tested (from 5.19 ± 0.69 to 7.77 ± 1.48 Hz, $p < 0.01$; Fig. 2B). In contrast, hypoosmotic stimulation (−25 mosmol kg^{-1}) caused a significant decrease in the rate of firing of 27 cells tested (from to 5.19 ± 0.69 to 3.64 ± 0.55 Hz, $p < 0.01$; Fig. 2B). Linear regression analysis of these data revealed a positive relation between mean firing rate and osmolality in these cells with a slope of +0.18 Hz kg $mosmol^{-1}$ (data not shown).

Glutamatergic pathway from OVLT to SON

It has been demonstrated in rats that neurons in the OVLT send glutamatergic projections to the SON (Yang et al., 1994; Trudel and Bourque, 2003) and that osmotic stimulation of the OVLT can modulate the firing of SON neurons via glutamatergic synapses (Richard and Bourque, 1995). To confirm the existence of a functional glutamatergic connection between the OVLT and the SON in mice, we examined the effects of electrical stimulation of the OVLT on MNCs studied by whole-cell recording in angled horizontal slices of mouse hypothalamus bathed in ACSF containing 5 μM bicuculline. Single electrical stimuli (0.1–0.2 ms, 10–100 μA) delivered to the OVLT evoked an excitatory postsynaptic current (EPSC) in each of seven MNCs tested. The mean amplitude of the EPSC varied as a positive function of stimulus amplitude (Fig. 3A). Moreover, as shown in Fig. 3B, C, EPSC amplitude was reversibly reduced by bath

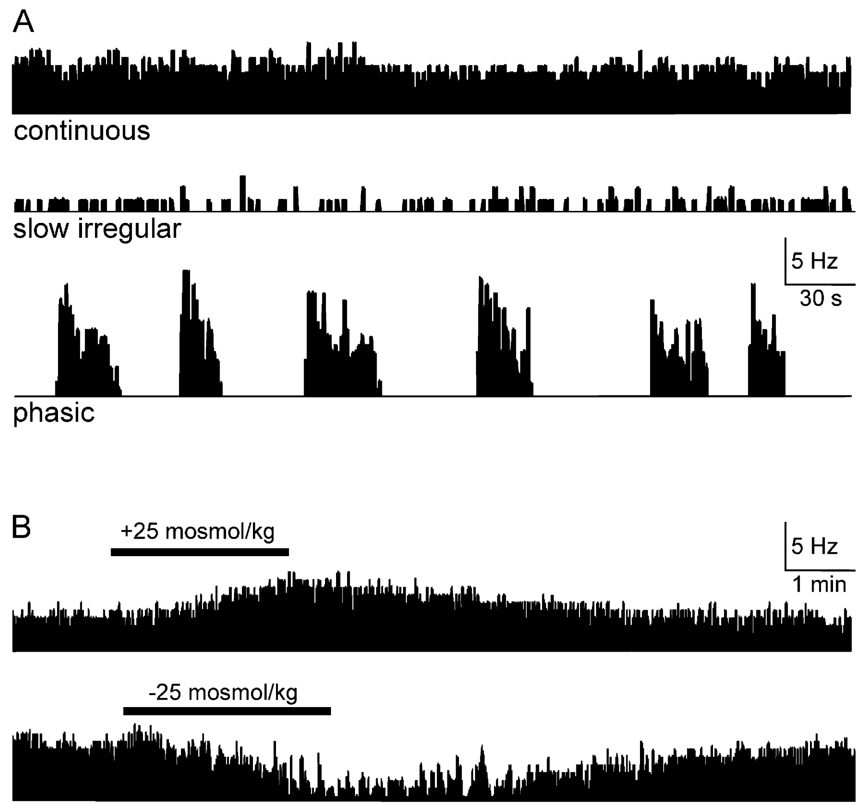

Fig. 2. Extracellular recordings from SON neurons in superfused explants. (A) Examples of spontaneous firing recorded from three separate SON neurons displayed as rate meter plots. The three panels show examples of continuous (top), slow irregular (middle) and phasic firing (bottom). (B) Effects of osmotic stimuli on the firing activity of two different SON neurons. Note that hypertonic (upper) and hypotonic stimuli (lower), respectively, evoke reversible excitatory and inhibitory responses from the cells.

application of KYN (5 mM, $61.09 \pm 7.11\%$ inhibition, $n = 4$), a broad-spectrum antagonist of ionotropic glutamate receptors.

Identification of MNCs in acutely dissociated mouse SON

Acutely dissociated preparations of SON have been used to study many of the intrinsic properties of MNCs in rats (Hussy et al., 1997; Zhang and Bourque, 2003). The MNCs isolated by this procedure retain their natural three-dimensional shape and are devoid of synapses (as opposed to those placed in tissue culture, which tend to flatten and to re-establish synaptic connectivity), and can be easily identified by their characteristic large size (Oliet and Bourque, 1992). To determine if MNCs acutely isolated from the dissociated mouse SON can also be identified by size alone, we performed a morphometric analysis of cells examined in preparations stained using a cocktail of antibodies directed at both AVP and OXT to selectively label all MNCs (see "Methods"). As illustrated in Fig. 4A, positive staining could be seen in large cells isolated by our procedure, whereas smaller cells tended to be immuno-negative. Analysis of 500 cells revealed that the mean CSA of MNCs ($206 \pm 3\,\mu m^2$, $n = 194$) is significantly greater than that of non-MNCs ($109 \pm 2\,\mu m^2$, $n = 306$, $p < 0.001$). Moreover, as illustrated by the frequency distribution shown in Fig. 4B, 96.4% of the cells having a CSA greater than $160\,\mu m^2$ (i.e. those displaying a minimum diameter of $14.3\,\mu m$) were found to be MNCs.

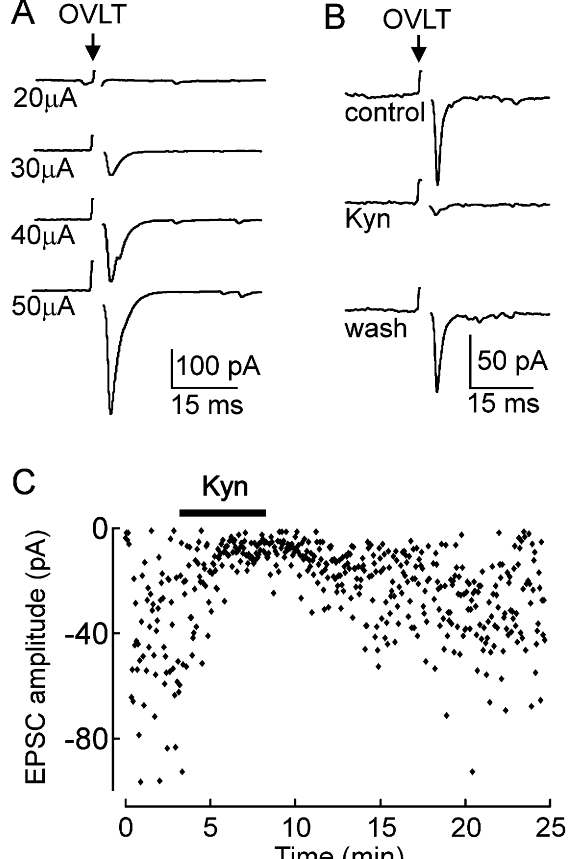

Fig. 3. Effects of OVLT stimulation on SON neurons. (A) Whole-cell voltage-clamp recordings from an SON neuron showing current responses (each trace is an average of six responses) to electrical stimuli (indicated at left) of increasing amplitude applied to the OVLT (arrow) in an angled horizontal slice of mouse hypothalamus. (B) Examples of EPSCs evoked in an SON neuron by OVLT stimulation (arrow) in the absence (control, wash) and presence of 5 mM KYN. Each trace is an average of six consecutive responses. (C) Graph plotting the amplitudes of individual EPSCs recorded from an SON neuron in response to single stimuli (50 μA, 0.1 ms) applied to the OVLT before, during and after bath application of KYN (Bar, 5 mM).

Effects of osmotic stimulation on acutely isolated mouse MNCs

Whole-cell current-clamp recordings were used to examine the effects of osmotic stimuli delivered to putative MNCs isolated from mouse SON. As illustrated in Fig. 5A, application of a hyperosmotic stimulus ($+60\,\text{mosmol}\,\text{kg}^{-1}$) caused a gradual depolarization of the membrane (from -58.4 ± 2.3 to -53.1 ± 2.3 mV, $n = 16$, $p<0.001$), a decrease in membrane input resistance (from 1023 ± 102 to 874 ± 81 MΩ, $n = 14$, $p<0.001$) and an increase in the cell firing rate (from 0.51 ± 0.19 to 0.83 ± 0.30 Hz, $n = 11$, $p<0.01$). In contrast, cells placed in a hypoosmotic environment ($-40\,\text{mosmol}\,\text{kg}^{-1}$) displayed a gradual membrane hyperpolarization (from -58.4 ± 2.3 to -61.1 ± 3.8 mV, $n = 13$, $p<0.001$), an increase in input resistance (from 1023 ± 102 to 1118 ± 238 MΩ, $n = 10$, $p<0.05$) and a decrease in firing rate (from 0.51 ± 0.19 to 0.35 ± 0.16 Hz, $n = 9$, $p<0.05$; Fig. 5B). As expected, the effects of hyperosmotic and hypoosmotic stimulation were, respectively, accompanied by significant decreases ($-12.7\pm2.0\%$, $n = 5$, $p<0.05$) and increases ($+15.3\pm2.2\%$, $n = 5$, $p<0.05$) in steady-state volume. As shown in Fig. 5C, the amplitude of the changes in membrane voltage observed in response to hyper- or hypoosmotic stimulation varied as a function of baseline voltage. Linear regression of the data sets showed extrapolated reversal potentials near -35 mV for both the hyperpolarizing effects of hypotonicity and the depolarizing effects of hypertonicity.

Discussion

In this study we sought to determine if specific features of MNCs that have been reported to contribute to the osmotic control of the hypothalamo-neurohypophysial system in rats are also expressed in the mouse. Although slight differences were found between the MNCs present in these two species of rodent, our study reveals that many of the basic features of rat and mouse MNCs are qualitatively equivalent when examined in three commonly used in vitro preparations.

Distribution of MNCs in the mouse SON

The overall distribution of AVP and OXT neurons observed by immunohistochemical staining of coronal sections of hypothalamus was consistent with previous observations in various strains of rats (Swaab et al., 1975; Vandesande and Dierickx, 1975; Rhodes et al., 1981) and mice (Pow and

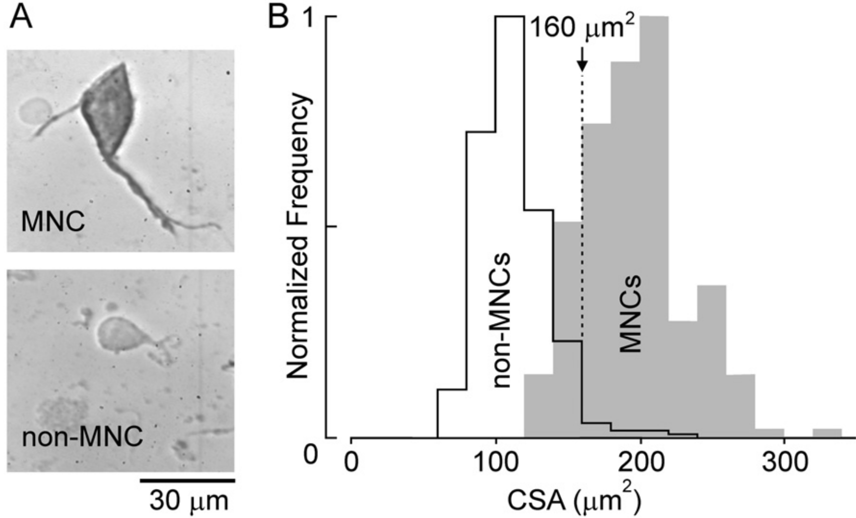

Fig. 4. Identification of MNCs in acutely dissociated SON of C57/BL mice. (A) Photomicrographs showing positive immunolabelling (upper) of a large cell by a cocktail of antibodies directed against AVP and OXT, and of a smaller unstained cell observed in the same preparation (lower). (B) Relative distribution (normalized frequency) of stained (MNCs, shaded area, $n = 194$) and unstained (non-MNCs, open area, $n = 306$) cells sorted according to size (CSA; bin width: $20\,\mu m^2$). Note that the vast majority of cells having a CSA $> 160\,\mu m^2$ (dashed line) are MNCs. Most of the cells isolated from the mouse SON showing a minimum diameter of 14.3 μm are thus MNCs.

Morris, 1989; Pirnik and Kiss, 2005). Specifically, OXT neurons in the mouse SON were located primarily in the dorsal portion of the SON, where they were often scattered between AVP-containing MNCs, and they were found in greater numbers in the anterior portion of the nucleus (Fig. 1). One notable feature that was observed in mouse SON was a relative scarcity of OXT-positive neurons. Indeed, OXT neurons accounted for only ~23% of MNCs in the SON of C57/BL mice. Thus, the overall proportion of AVP MNCs present in the mouse SON is even greater than in rats [e.g. ~69% in Wistar (Swaab et al., 1975), 60% in Brattleboro and 71% in Long-Evans (Rhodes et al., 1981)]. Based on these proportions, and the obvious scarcity of OXT neurons present in the ventral part of the SON (Fig. 1), it may be presumed that most electrophysiological recordings obtained from the ventral part of the SON are from AVP-releasing MNCs.

Activity of SON neurons in superfused explants of mouse hypothalamus

Extracellular recordings from SON neurons in superfused explants of mouse hypothalamus revealed that MNCs in this species display patterns of spontaneous action potential discharge that are strikingly similar to those observed in rats, including the ability to fire in a phasic pattern (for review see Poulain and Wakerley, 1982; Bourque and Renaud, 1990). In rats, the expression of phasic firing by MNCs has been shown to optimize excitation–secretion coupling from their peptide-releasing axon terminals in the neurohypophysis (Bicknell, 1988), and the emergence and modulation of this pattern is an important aspect of the physiological response to stimuli that regulate AVP release (Walters and Hatton, 1974; Brimble and Dyball., 1977; Wakerley et al., 1978). Although much has been learned concerning the basic cellular mechanisms involved in the generation and modulation of phasic firing (Li et al., 1995; Ghamari-Langroudi and Bourque, 2004; Brown and Bourque, 2006), the nature of the ion channel responsible for the activity-dependent plateau potential that assists firing during phasic bursts remains elusive even more than 20 years after the process was first described by intracellular recording (Andrew and Dudek, 1983). Preliminary data obtained by intracellular recording with sharp electrodes show that SON neurons in superfused

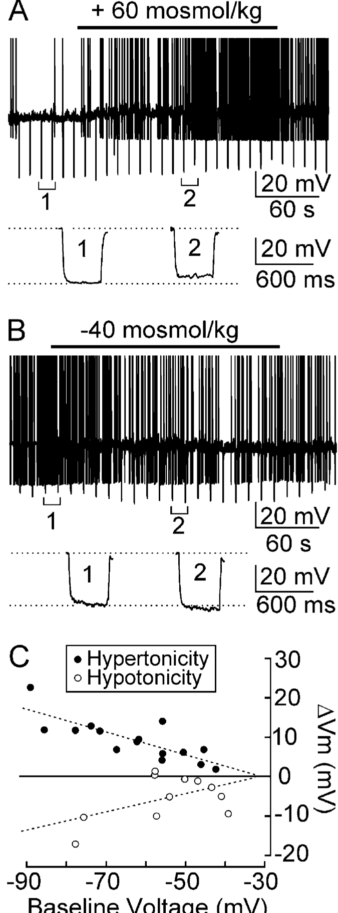

Fig. 5. Osmosensitivity of acutely isolated mouse MNCs. Whole-cell current-clamp recordings show the effects of hypertonic (A) and hypotonic (B) stimuli on two acutely isolated MNCs. In each panel, the top trace is the raw voltage record showing the entire experiment. The large upward transients in these records are action potentials (peaks not shown), whereas the downward-going transients are the electrotonic responses to hyperpolarizing current pulses (−50 pA, 500 ms) applied to monitor input resistance. The traces shown below each raw record are excerpts showing the average of two consecutive electrotonic voltage responses induced before (1) and during (2) the osmotic stimuli (positions shown by corresponding brackets). The baselines of the latter have been aligned (upper dashed lines) to highlight changes in amplitude (lower dashed lines). Note that the depolarizing effect of hypertonicity is associated with a decrease in resistance, whereas the hyperpolarizing effect of hypotonicity is associated with an increase in membrane resistance. (C) Graphs plotting the amplitude of changes in membrane voltage (ΔV_m) caused by hypertonic (filled circles) and hypotonic stimuli (open circles) as a function of baseline voltage. Note that the absolute magnitude of responses to both stimuli increases with hyperpolarization. Dashed lines are separate linear regressions through the data sets. Note that these plots converge near an extrapolated reversal voltage of −35 mV.

explants of mouse hypothalamus display spike depolarizing after-potentials, plateau potentials and after-discharge properties equivalent to those observed in rat MNCs (data not shown). The availability of mice in which specific ion channels have been altered or deleted should thus provide effective models in which this process can now be studied at a molecular level.

Osmosensitivity of mouse SON neurons

Previous studies in rat have shown that secretion of AVP and OXT into the circulation is induced by calcium influx through voltage-gated channels that are opened upon the arrival of action potentials into the axon terminals of MNCs in the neurohypophysis (for review see Bicknell, 1988; Fisher and Bourque, 2001). Calcium-dependent hormone secretion can also be induced by electrical stimulation of the isolated mouse neurohypophysis (Wolfe and Gainer, 1986). The presence of spontaneous electrical activity in mouse MNCs is therefore consistent with the active release of AVP that occurs under basal conditions in mice (Morris et al., 1999; Sharif-Naeini et al., 2006). In other species, this basal release of AVP has been shown to mediate significant antidiuresis at the kidney, thus permitting a bidirectional regulation control over renal water balance. Specifically, enhanced water retention can be promoted by increasing AVP release (e.g. under hyperosmotic conditions), and diuresis can be induced by suppressing basal AVP secretion (e.g. under hypoosmotic conditions) (Dunn et al., 1973; Robertson et al., 1976). Our recordings from osmotically stimulated MNCs in superfused explants suggest a similar, bidirectional, modulation of AVP release in mice. Indeed, the spontaneous electrical activity of mouse MNCs expressed at the osmotic set point could be significantly reduced or enhanced by hypoosmotic and hyperosmotic stimuli, respectively (Fig. 2). Interestingly, the overall osmoresponsiveness of SON MNCs in in vitro explants of mouse hypothalamus (+0.18 Hz kg mosmol^{-1}) was similar to that of rat MNCs measured in vivo (+0.25 Hz kg mosmol^{-1}; Bourque, 1998), suggesting that conserved mechanisms may couple osmotic stimuli to changes in firing rate in these species.

Mouse SON neurons receive glutamatergic synaptic inputs from the OVLT

Studies in a variety of mammals have shown that the osmotic regulation of AVP release by MNCs is mediated in part by afferents arising from various structures associated with the lamina terminalis (for review see Bourque et al., 1994; McKinley et al., 2004). Although it is likely that several direct and indirect excitatory and inhibitory projections arising from various nuclei participate in this process, experiments in superfused hypothalamic explants have shown that glutamatergic synaptic connections originating specifically from osmosensitive neurons in the OVLT play a crucial role in the osmotic control of electrical activity of MNCs in the rat SON (Richard and Bourque, 1995). Our experiments on angled horizontal hypothalamic slices confirmed that electrical stimulation of the OVLT can also elicit KYN-sensitive (i.e. glutamatergic) synaptic responses in mouse SON neurons. A recent study in rat has shown that these synapses can undergo activity-dependent plasticity (Panatier et al., 2006). The availability of numerous lines of mice expressing altered glutamate receptors and modified signalling systems should thus facilitate the analysis of the functional impact of short-term and long-term plasticity in this pathway in future studies.

Mouse MNCs are intrinsically osmosensitive

Previous work in rat has shown that the osmoresponsiveness of SON neurons in situ results from a combination of factors that includes changes in synaptic drive mediated by inputs arising in the lamina terminalis and locally generated changes in membrane potential (Leng et al., 1989). Factors that might contribute to the acute local osmotic control of membrane potential in rat MNCs include: (i) changes in glycine receptor activation following altered taurine release from surrounding glial cells (Hussy et al., 2000); (ii) changes in the activity of various types of K^+ channels (Han et al., 2003; Liu et al., 2005); and (iii) changes in non-selective cation current mediated by mechanosensitive channels (Voisin and Bourque, 2002). Mechanisms that are intrinsically generated by the MNCs themselves are easily studied in neurons acutely isolated from the SON. Our analysis showed that large neurons ($>14\,\mu m$ diameter) acutely isolated from the SON of the mouse can be identified as MNCs with reasonable accuracy.

Whole-cell recording experiments from isolated mouse MNCs confirmed that these neurons are intrinsically osmosensitive, being depolarized and excited by hypertonic stimuli and hyperpolarized and inhibited by hypoosmotic stimuli (Fig. 5). Our analysis showed further that these effects were accompanied respectively by decreases and increases in membrane resistance. This observation indicates that, under our experimental conditions, hypertonicity increased the activity of ion channels whereas hypotonicity suppressed channel activity. Moreover, the voltage-dependence of osmotically evoked responses measured in current-clamp indicated that the responses were mediated by ion channels capable of flowing a current reversing near $-35\,mV$, a value consistent with responses observed under voltage-clamp in the same preparation (Sharif-Naeini et al., 2006). Thus, the intrinsic osmosensitivity of mouse MNCs appears to be mediated in large part by the acute regulation of non-selective cation channels. Mouse genetic models have already provided some information concerning the molecular players that may be involved in osmoregulation (Liedtke and Friedman, 2003) and osmosensory transduction (Ciura and Bourque, 2006; Sharif-Naeini et al., 2006). Further use of mouse models is likely to increase our mechanistic understanding of these important physiological processes.

Concluding statement

Overall, these data indicate that MNCs in the mouse SON display several electrophysiological features that are comparable to those reported previously in the rat. The availability of several genetically modified strains of mice should therefore allow us to gain a deeper understanding of the molecular mechanisms that underlie the osmotic control of electrical activity and hormone release by the hypothalamo-neurohypophysial system.

Abbreviations

ACSF	artificial cerebrospinal fluid
AVP	arginine vasopressin
CSA	cross-sectional area
EPSC	excitatory postsynaptic current
KYN	kynurenic acid
MNC	magnocellular neurosecretory cell
NGS	normal goat serum
OXT	oxytocin
OVLT	organum vasculosum laminae terminalis
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PVN	paraventricular nucleus
SON	supraoptic nucleus

Acknowledgements

We thank Dr. H. Gainer (N.I.H., Bethesda, MD) for supplying the primary antibodies used in this study. Our work was supported by Canadian Institutes of Health Research (CIHR) operating grants MOP-9939 and MOP-82818 to C.W. Bourque who was also recipient of a CIHR Senior Investigator Award and a James McGill Research Chair. E.T. was recipient of a Doctoral Research Award from the Heart and Stroke Foundation of Canada (HSFC). R. Sharif-Naeini was recipient of Doctoral Awards from CIHR and HSFC. S. Ciura was recipient of a Doctoral Award from the Canada Graduate Scholarships Programme. T.J. Stachniak was recipient of a Doctoral Award from the CIHR.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 21

Effects of oxytocin on GABA signalling in the foetal brain during delivery

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Abstract: Oxytocin (OXT) exerts multiple effects in the adult central nervous system. However, little is known about the effects of OXT on foetal neurons during delivery, at the time when a surge of OXT occurs. In a recent study, the effects of OXT on gamma-aminobutyric acid (GABA) signalling have been reported in foetal and newborn rats. In the immature rat hippocampal and neocortical neurons at birth, endogenous OXT induced a switch in the action of GABA from excitatory to inhibitory. This excitatory-to-inhibitory switch was caused by a switch in the polarity of the GABAergic responses from depolarizing to hyperpolarizing, reflecting a decrease in the intracellular chloride concentration. The effects of OXT were mimicked and occluded by bumetanide, a selective blocker of the chloride co-transporter NKCC1, suggesting that the effects of OXT involve inhibition of NKCC1. Neuronal death caused by anoxic–aglycaemic episodes was substantially delayed in the foetal hippocampus by endogenous OXT. These findings suggest that OXT plays important role in the preparation of the foetal brain to delivery.

Keywords: oxytocin; GABA; foetus; neonate; hippocampus; cortex; delivery

GABA in the immature brain

Gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the adult brain (Farrant and Kaila, 2007). However, early in development (including foetal and postnatal periods), GABA has a depolarizing and excitatory action on immature neurons. The excitatory action of GABA is due to an elevated $[Cl^-]_i$ and thus depolarized value of the reversal potential of GABA-mediated responses (E_{GABA}) (Ben-Ari et al., 1989; Serafini et al., 1995; Chen et al., 1996; Owens et al., 1996; Rivera et al., 1999) (for a review see, Ben Ari et al., 2007). Developmental changes in two cation-chloride co-transporters, chloride loader NKCC1 and chloride extruder KCC2 — play a pivotal role in the developmental changes in $[Cl^-]_i$. In cortical neurons NKCC1 is a membrane transport protein that mediates chloride uptake across the plasma membrane internalizing one Na^+, one K^+ and 2 Cl^- in electro neutral coupled fashion (Payne et al., 2003). NKCC1 does not use ATP but operates using the electrochemical gradient for Na^+ and K^+ produced by the Na^+, K^+-ATPase. High expression of NKCC1 in immature neurons maintains high $[Cl^-]_i$ (Clayton et al., 1998; Rohrbough and Spitzer, 1996; Plotkin et al., 1997; Fukuda et al., 1998; Delpire, 2000; Sung et al., 2000; Mikawa et al., 2002; Li et al., 2002; Wang et al., 2002; Payne et al., 2003; Yamada et al., 2004; Dzhala et al., 2005). KCC2 is the principal transporter for Cl^- extrusion from neurons. KCC2 extrudes K^+ and Cl^- using

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DOI: 10.1016/S0079-6123(08)00421-4

the electrochemical gradient for K^+. Cl^- extrusion is weak in immature neurons and increases with neuronal maturation (Deisz and Lux, 1982; Misgeld et al., 1986; Thompson et al., 1988a, b; Thompson and Gähwiller, 1989; Luhmann and Prince, 1991; Zhang et al., 1991; Jarolimek et al., 1999; Lu et al., 1999; Rivera et al., 1999; Wang et al., 2002; Shimizu-Okabe et al., 2002; Yamada et al., 2004; Khirug et al., 2005). Thus, reciprocal developmental changes in the expression of these two main chloride co-transporters, a decrease in NKCC1 and an increase in KCC2, are pivotal in a developmental decrease of $[Cl^-]_i$ in the cortical neurons.

Depolarizing GABA often is excitatory, triggering action potentials in immature neurons. In addition to this direct excitatory action, depolarizing GABA may also activate voltage-gated calcium channels to increase intracellular calcium concentration, attenuate voltage-dependent magnesium block and potentiate the activity of *N*-methyl-D-aspartate (NMDA) channels (for a review, see Ben-Ari et al., 1997, 2007). Depolarizing and excitatory GABA plays an important role in the generation of particular patterns of activity expressed in the immature brain. Thus, depolarizing GABA is instrumental in the generation of giant depolarizing potentials in the neonatal rat hippocampus (Ben-Ari et al., 1989; Khazipov et al., 1997; Leinekugel et al., 1997; Khazipov et al., 2004). Excitatory GABA also acts as a trophic factor during nervous system development influencing events such as proliferation, migration, differentiation, synapse maturation and cell death (Owens and Kriegstein, 2002; Represa and Ben-Ari, 2005). The importance of depolarizing GABA signalling in the immature brain is reinforced by the fact that GABAergic synapses are established first on cortical neurons, prior to glutamatergic synapses so that in the rat at birth many neurons receive predominantly GABAergic input (Tyzio et al., 1999; Khazipov et al., 2001).

Perinatal switch in the action of GABA from excitatory to inhibitory

Near term changes in GABA signalling were studied in CA3 pyramidal neurons of the rat hippocampus (Tyzio et al., 2006). In keeping with the results of previous studies, during foetal (E18–19) and postnatal periods (P1–5), activation of $GABA_A$ receptors increased the firing of action potentials in the majority of CA3 pyramidal cells (Ben-Ari et al., 1989; Leinekugel et al., 1997; Rivera et al., 1999). However, during a brief period extending from E20 to the day of birth (P0), the proportion of cells excited by GABA sharply decreased. The loss of this excitatory effect of GABA peaked at E21 (shortly before delivery). Subsequently, the hypothesis was explored that the perinatal loss of the excitatory action of GABA is due to a change in E_{GABA}. To test this, the GABA driving force (DF_{GABA}) and resting membrane potential (E_m) were estimated using cell-attached recordings of single $GABA_A$ and NMDA channels, respectively (Serafini et al., 1995; Leinekugel et al., 1997; Tyzio et al., 2003) (Fig. 1B–D). Considerable advantage of cell-attached recordings is that they affect neither E_{GABA} nor E_m the estimation of which can be compromised in small neurons using conventional intracellular or whole-cell recording techniques (Barry and Lynch, 1991; Tyzio et al., 2003). DF_{GABA} was strongly depolarizing in the foetal and postnatal periods (Fig. 1D) but negatively shifted during a brief near-term period (from E20 to P0), switching to completely hyperpolarizing at term (Fig. 1D). Thus, the disappearance of GABA-mediated excitation at term (Fig. 1A) coincides with a switch in polarity of GABA signals from depolarizing to hyperpolarizing. During the early foetal and postnatal periods E_m was about -80 mV but near term there is a small hyperpolarizing shift to -85 mV. Knowing DF_{GABA} and E_m, E_{GABA} was calculated ($E_{GABA} = DF_{GABA} + E_m$) and it was found that E_{GABA} switches from -40 mV at E18 to -92 mV at E21 and then returns to depolarizing values shortly after birth (Fig. 1F). This corresponds to a decrease in $[Cl^-]_i$ from 18 to 4 mM (Fig. 1F). In keeping with the electrophysiological observations, using a multibeam two-photon microscope to monitor $[Ca^{2+}]_i$ it was found that during delivery, the ability of GABA to increase $[Ca^{2+}]_i$ characteristic of depolarizing GABA (Connor et al., 1987; Yuste and Katz, 1991; LoTurco et al., 1995; Leinekugel et al., 1997; Garaschuk et al., 1998), is significantly reduced.

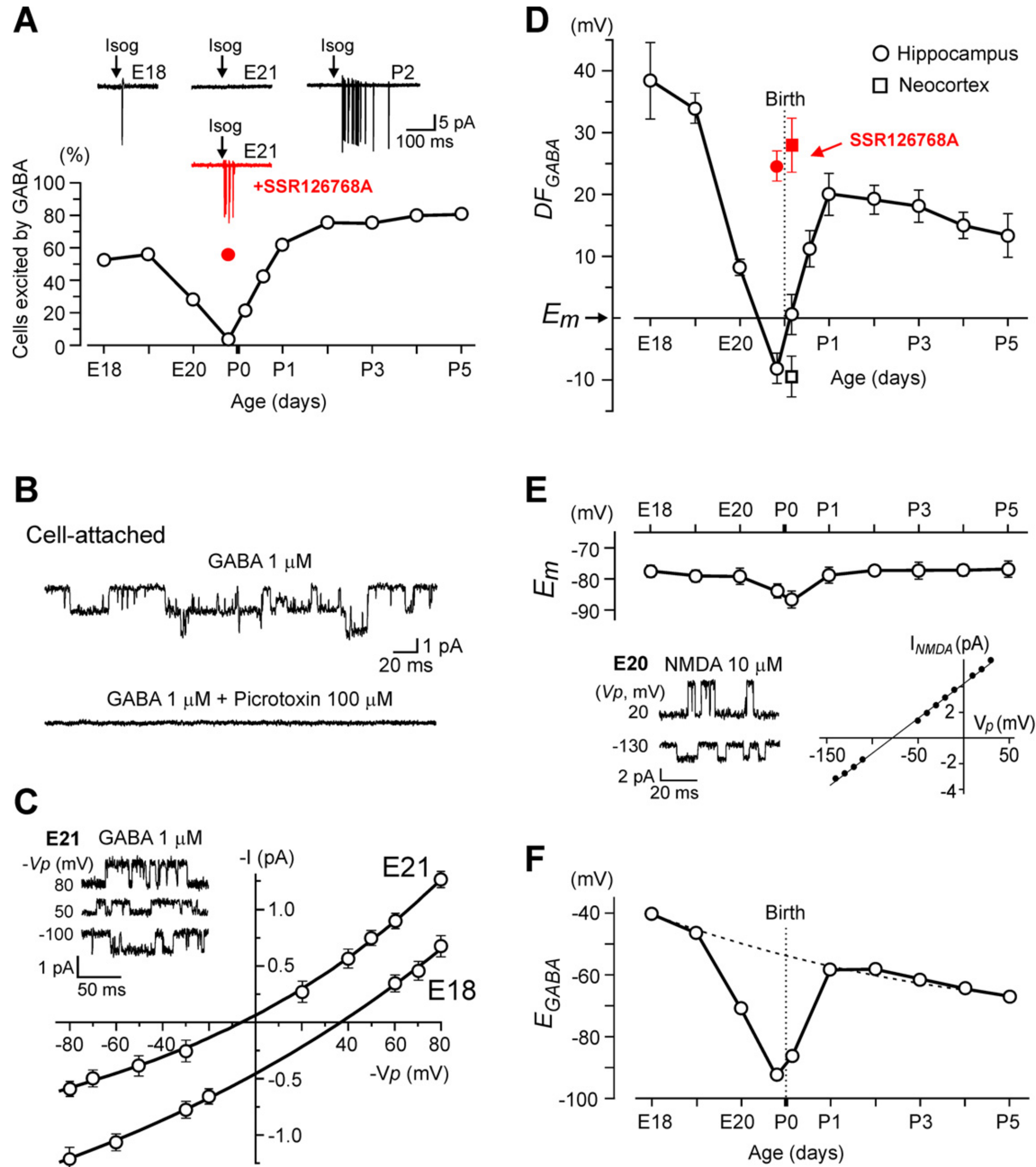


Fig. 1. Transient perinatal loss of the $GABA_A$-mediated excitation. (A) Responses of CA3 pyramidal cells recorded in cell-attached mode to the $GABA_A$ agonist isoguvacine. Below, summary plot of the proportion of cells excited by isoguvacine during the perinatal period. Note transient loss of the excitatory effect of isoguvacine near term. Red code corresponds to the foetuses whose mothers received the OXT receptor antagonist SSR126768A. [E, embryonic; E21 corresponds to the early phase of delivery; P, postnatal; P0 is the day of birth; pooled data from 146 neurons]. (B) Cell-attached recordings of single $GABA_A$ channels with 1 μM of GABA in patch pipette (upper trace); the channels were not observed in the presence of the $GABA_A$ antagonist picrotoxin (100 μM; lower trace). (C) I–V relationships of the currents through $GABA_A$ channels in two cells, at E21 and E18; their reversal potential corresponds to the $GABA_A$ driving force (DF_{GABA}). (D) Summary plot of the age-dependence of DF_{GABA} inferred from single $GABA_A$ channels recordings [mean ± SEM; 209 CA3 pyramidal cells (○) and 17 neocortical pyramidal cells (□); 6–24 patches for each point]. Red code — pretreatment with SSR126768A ($n = 25$ hippocampal and 9 neocortical patches). (E) Age-dependence of the resting membrane potential (E_m) of CA3 pyramidal cells inferred from the reversal of single NMDA channels recorded in cell-attached mode ($n = 84$ cells; 4–12 patches for each point). (F) Age-dependence of the $GABA_A$ reversal potential ($E_{GABA} = E_m + DF_{GABA}$). Note a transient hyperpolarizing shift of E_{GABA} near birth. Adapted with permission from Tyzio et al., 2006.

Involvement of oxytocin in the perinatal GABA switch

The near-term switch in GABA action may reflect a developmental phenomenon or, alternatively, it could be related to parturition. However, this phenomenon was not observed in foetal neurons grown in culture (Chen et al., 1996; Owens et al., 1996). Therefore, the perinatal switch is likely related to parturition and in particular to maternal hormones released during delivery. Parturition is initiated by a massive release of oxytocin (OXT) (Russell et al., 2003). In addition to its pivotal role in parturition, there are also indications that OXT exerts multiple effects in the adult central nervous system (Argiolas and Gessa, 1991; Raggenbass, 2001; Gimpl and Fahrenholz, 2001; Tomizawa et al., 2003; Kosfeld et al., 2005; Huber et al., 2005; Theodosis et al., 2006). Therefore, a hypothesis was explored that OXT is responsible for the hyperpolarizing switch in action of GABA in foetal neurons during delivery. During cell-attached recordings from slices at E18 and P2, applications of OXT induced a negative shift in DF_{GABA} (Fig. 2C, D) and suppressed GABA-mediated excitation (Fig. 2B). The effects of OXT were prevented by the selective OXTR antagonist atosiban (Fig. 2D). At term, application of OXT did not cause a significant effect on the hyperpolarizing DF_{GABA} (Fig. 2D) suggesting that the effects of exogenous OXT are occluded by the endogenous hormone. In keeping with this hypothesis, OXTR antagonist atosiban switched DF_{GABA} from hyperpolarizing to depolarizing. Effects of OXT on $[Cl^-]_i$, were also measured using two-photon chloride imaging in slices loaded with a chloride-sensitive dye *N*-(ethoxycarbonyl-methyl)-6-methoxyquinolinium bromide (MQAE, Fig. 2E, F) (Marandi et al., 2002). In E18 and P4 slices, OXT produced a significant increase in the baseline fluorescence signal in half of the imaged cells indicating a strong reduction of $[Cl^-]_i$ (Fig. 2F) and the effect was prevented by atosiban. Furthermore, incubation of P4 slices with OXT reversed the effect of isoguvacine on spontaneous calcium events. Thus, the effects of OXT fully match the changes in GABA signalling occurring at term. This indicates that OXT is sufficient to trigger changes of $[Cl^-]_i$. To determine whether endogenous OXT is necessary for the near-term switch in the action of GABA, pregnant rats were treated per orally with the selective OXT receptor antagonist SSR126768A (Serradeil-Le Gal et al., 2004) and the consequences measured at term. Cell-attached recordings revealed major differences between treated and age matched control pups including strongly depolarizing values of DF_{GABA} (Fig. 1D, grey circle) and high proportion of cells excited by GABA (Fig. 1A, grey circle). Similar effects were also observed in neocortical neurons (Fig. 1D, squares). Thus, maternal OXT is necessary and sufficient to trigger the near-term switch in GABA action in the hippocampus and neocortex.

Source of oxytocin in a slice preparation

These findings, and in particular the effects of atosiban in the in vitro experiments, indicate that ambient OXT is present in the slice preparation. This is an intriguing finding because slices are continuously perfused with OXT-free solution, and OXT should have been rapidly washed out from the extracellular space. Therefore, the question arises what is the source of OXT in the slice? Possible explanations involve synthesis and release of OXT by the hippocampal neurons or a passive diffusion of OXT from the blood vessels, which may contain large amounts of endogenous hormone. Staining against OXT antibodies failed to reveal detectable amounts of OXT in the hippocampal neurons and glial cells, making the former hypothesis unlikely. To test the second hypothesis, prior to slice preparation, E21 foetuses were intracardially perfused with artificial cerebrospinal fluid to wash out endogenous hormone. This procedure produced a shift of DF_{GABA} from hyperpolarizing to depolarizing (Fig. 3). Furthermore, addition of OXT at the end of the perfusion restored hyperpolarizing values of DF_{GABA} (Fig. 3). Bath application of atosiban to the slices obtained from the foetuses that have been initially intracardially perfused with ACSF and then with OXT induced depolarizing shift of DF_{GABA} (Tyzio et al., 2006). These results are compatible with the

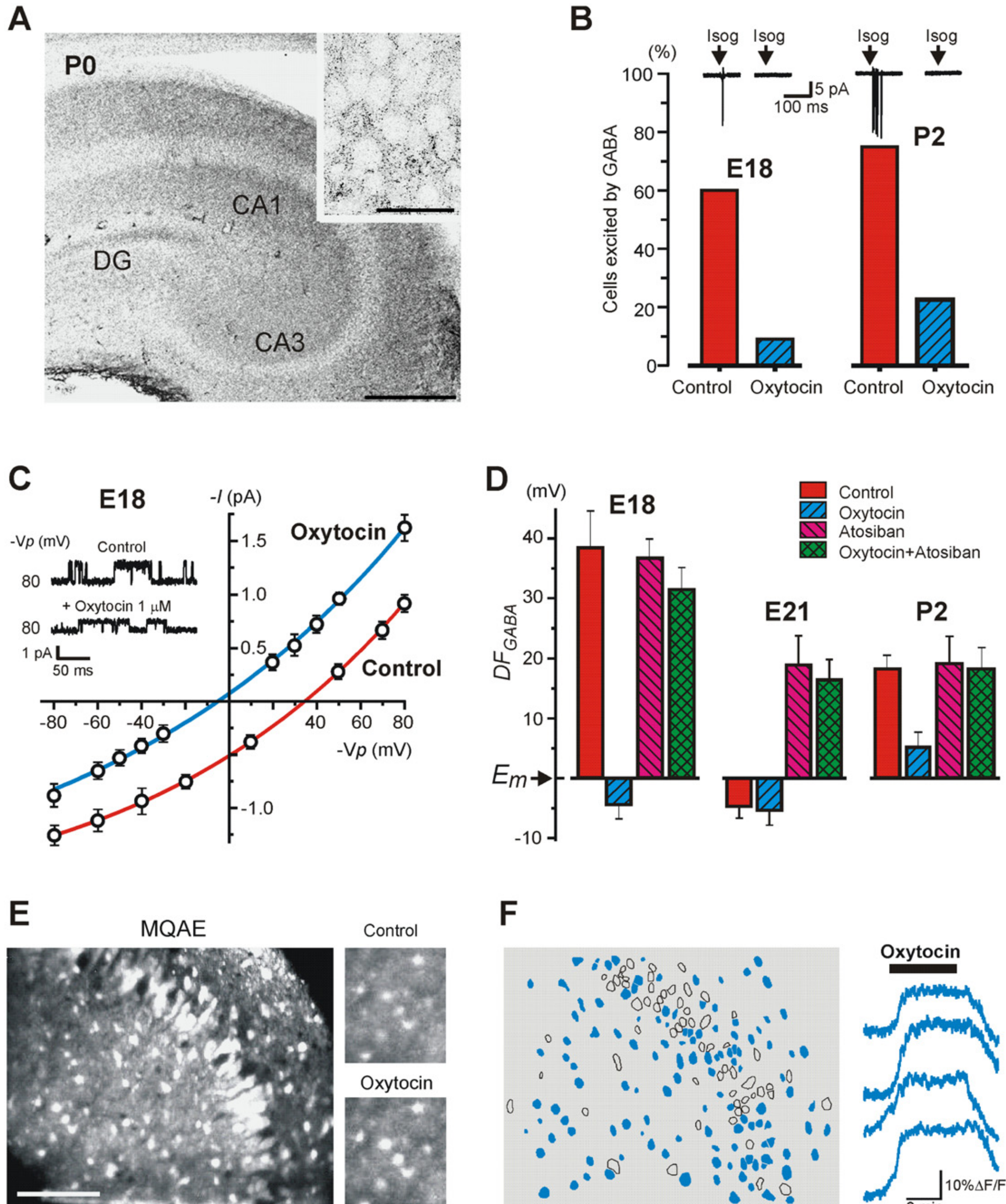


Fig. 2. Oxytocin causes a switch in $GABA_A$ signalling from depolarizing to hyperpolarizing at birth. (A) Oxytocin-receptor immunostaining of a P0 rat hippocampus; inset: CA3 pyramidal cells layer. Bars: 200 and 20 μm. (B) Histograms of the proportion of cells excited by brief application of isoguvacine (Isog) in control and in the presence of OXT (1 μM) at E18 and P2 ($n = 57$ cells). (C) I–V relationships of the currents through single $GABA_A$ channels recorded from two CA3 pyramidal cells at E18 in control and after addition of OXT (1 μM). (D) Histograms of DF_{GABA} measured at E18, E21 and P2 in control conditions and in the presence of OXT (1 μM), antagonist of OXT receptors atosiban (1–5 μM) and OXT + atosiban ($n = 187$ cells). (E) Two-photon imaging of $[Cl^-]_i$ in P4 hippocampal slice loaded with a Cl^- indicator MQAE. Scale bar: 100 μm. Right panel: $[Cl^-]_i$ fluorescence change produced by OXT application. Note that the intensity of the fluorescence signal increases, and $[Cl^-]_i$ decreases, in the illustrated region. (F) Automatically detected contours of the cells imaged in (E) indicating the distribution of cells in which OT application produced a significant decrease in $[Cl^-]_i$ (blue filled contours). On the right, chloride fluorescence changes in four representative neurons. Time resolution: 100 ms/frame. Adapted with permission from Tyzio et al., 2006. (See Color Plate 21.2 in color plate section.)

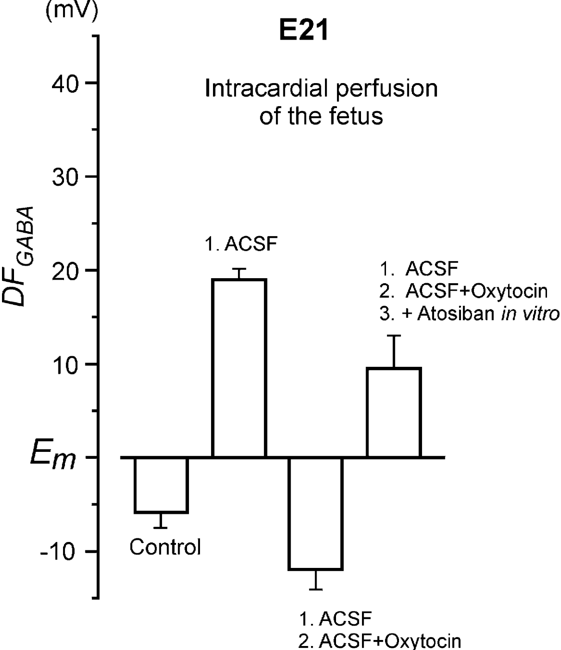


Fig. 3. Origin of endogenous oxytocin in the slice preparation. Summary histogram showing $GABA_A$ driving force (DF_{GABA}) deduced from cell-attached single $GABA_A$ channels recordings in CA3 pyramidal cells of E21 foetal rat hippocampal slices. Note that DF_{GABA} is negative in control (non-perfused) animal. Prolonged (30 min) intracardial perfusion of foetuses with ACSF at term produces strong depolarizing shift of DF_{GABA}. Addition of 1 μM-OXT at the end of perfusion restored hyperpolarizing values of DF_{GABA}. Bath application of atosiban (5 μM) to the slices obtained from the foetuses that have been initially intracardially perfused with ACSF and then with OXT (1 μM, at the end of perfusion), induces depolarizing shift of DF_{GABA}. Pooled data from 45 cells. These results are compatible with the hypothesis that residual maternal OXT remaining in foetal blood vessels provides tonic activation of OXT receptors in a slice. Adapted with permission from Tyzio et al., 2006.

hypothesis that residual OXT remaining in foetal blood vessels provides tonic activation of OXTRs in a slice. They also raise an important and more general methodical point when using slice preparations for a physiological experiment. It could be suggested that other hormones may have pharmacokinetics similar to OXT and account for the physiological changes observed in the slices prepared from the experimental animal.

Origin of oxytocin in the foetal brain: maternal or foetal?

In the foetal brain, OXT may be provided both by the mother and the foetus, and the maternal and foetal contributions may differ in rodents and humans. Several lines of evidence suggest that in the rat the principal source of OXT is maternal with only a limited contribution of foetus. Indeed, hypothalamo-neurohypophysial system in the rat develops mainly postpartum (Choy and Watkins, 1979; Lipari et al., 2001, but also see Schriefer et al., 1982; van der Sluis et al., 1986). Quantitative radioimmunoassay analysis of precursor, intermediate and completely processed forms of OXT revealed that OXT precursors are synthesized during the foetal period. However, production of fully functional amidated OXT starts only at term and increases rapidly upon postnatal development (Alstein et al., 1988). A more recent study using immunohistochemical and real time-polymerase chain reaction (RT-PCR) analysis has reported that in rats, OXT synthesis starts later, at P2 (Lipari et al., 2001). Thus, the onset of foetal OXT production does not match with the perinatal OXT-mediated changes in GABA signalling which is already half-maximal by E20 (Tyzio et al., 2006).

On the other hand, maternal OXT, secreted from nerve terminals of OXT neurons in the posterior pituitary gland, is important in the timing of birth. At birth, OXT cells fire in coordinated bursts that generate pulsatile OXT secretion to promote rhythmic contraction of the uterus (Russell et al., 2003). Therefore maternal OXT is perfectly suited to alert the foetal brain to the imminent onset of parturition before the day of birth. In order to reach the foetal brain, maternal OXT must cross placenta and the blood–brain barrier. Recently, it has been suggested (Brown and Grattan, 2007) that it is unlikely that maternal OXT would reach the foetal brain because placental leucine aminopeptidase, which is synthesized by syncytiotrophoblasts would degrade it (Kobayashi et al., 2004). However, administration of OXT to pregnant rats induces the inhibitory GABA switch in the foetal neurons suggesting that maternal OXT indeed reaches the foetal brain, crossing both placenta and the immature blood–brain barrier (Tyzio et al., 2006).

This is in keeping with an observation made using in vitro dually perfused isolated cotyledons from term human placenta, which demonstrated that maternal OXT easily crosses the placenta by simple diffusion to reach the foetus and vice versa, and that there is little evidence of placental metabolism and degradation of OXT (Malek et al., 1996). Taken together, these results indicate that OXT in the foetal brain is mainly provided by the mother. However, additional foetal contributions remain to be determined. This could be achieved by comparison of DF_{GABA} in homozygote and heterozygote foetuses of pregnant OXT knockout mice.

Recently, the intriguing hypothesis had been suggested that during delivery, hypoxia induced release of cortisol triggers release of foetal OXT that would drive a perinatal inhibitory switch in the GABA signalling (Carbillon, 2007). This hypothesis was tested experimentally by studying the glucocorticoid modulation of GABA signalling in 1-day-old rats (Tyzio et al., 2007). The $GABA_A$ driving force (DF_{GABA}) was measured in CA3 pyramidal cells using cell-attached recordings of single $GABA_A$ channels. In hippocampal slices prepared from control rat pups, DF_{GABA} was strongly depolarizing (+20 mV), that is in agreement with the results of previous study (Tyzio et al., 2006). In slices prepared from rat pups administered intraperiotoneally with the cortisol analogue methylprednisolone, the values of DF_{GABA} were also strongly depolarizing and not significantly different from control values (+20 mV). Thus, even if a cortisol-induced release of OXT from the newborn rat hypothalamus was confirmed, it is insufficient to trigger the switch in the GABA action. Whether cortisol could directly affect GABA actions was also examined. In the presence of methylprednisolone, DF_{GABA} was not different from control values (+18 mV). Two-photon imaging of intracellular chloride in hippocampal pyramidal cells using chloride-sensitive fluorescent dye MQAE also failed to reveal any substantial change in the intracellular chloride concentration in response to methylprednisolone. Taken together, these findings indicate that the hypothesized cortisol-induced release of foetal OXT, or cortisol itself, does not significantly contribute to the inhibitory switch of GABA signalling at birth, and further supports a maternal origin of OXT in foetal rat brain.

In the human brain, the contribution of foetal OXT may be more significant. Compared with altritial rats, the human brain is more advanced in development at term, including its hypothalamo-neurohypophyseal system. In humans, OXT concentrations are higher in the umbilical artery (15–40 pg/ml) than in the umbilical vein (4–12 pg/ml) at term (Chard et al., 1971; Dawood et al., 1978; Otsuki et al., 1983; Patient et al., 1999). In addition, no OXT was detected in either arterial or venous cord blood of anencephalic newborns (Chard et al., 1971; Otsuki et al., 1983). These observations suggest that human foetuses produce OXT at term. Moreover, although controversial (Otsuki et al., 1983; Patient et al., 1999) it has been suggested that foetal OXT participates in parturition (Chard et al., 1971; Dawood et al., 1978; Fuchs et al., 1982). What triggers release of OXT in human foetus at birth is unknown. A common denominator might be the stress and anoxia associated with delivery, or the reduction in placental blood flow during contractions.

Oxytocin and NKCC1

What are the mechanisms underlying the OXT-mediated reduction of $[Cl^-]_i$ at term? Two possible yet non-exclusive mechanisms involve suppression of chloride loading and stimulation of chloride extrusion. As discussed above, $[Cl^-]_i$ is mainly maintained in the immature cortical neurons by highly expressed chloride loader NKCC1, whereas the principal chloride extruder is expressed only starting from the second postnatal week (Rivera et al., 1999; Payne et al., 2003; Yamada et al., 2004; Dzhala et al., 2005). Under such circumstances, application of Occam's razor would predict that an OXT-mediated reduction of $[Cl^-]_i$ involves suppression of NKCC1. Another "economic" argument favouring this hypothesis is the high energetic cost of NKCC1. Although NKCC1 does not directly consume energy, it uses the electrochemical gradient of sodium which is maintained by the Na, K-ATPase. Therefore,

activation of a chloride-extrusion mechanism, without suppression of NKCC1 activity, would multiply the energy cost of $[Cl^-]_i$ reduction and increase vulnerability to metabolic deprivations such as hypoxia–aglycaemia, which is not the case (see below).

Several lines of experimental evidence support the idea that the effect of OXT on $[Cl^-]_i$ involves down-regulation of NKCC1. It was shown that the highly selective NKCC1 antagonist bumetanide mimics the effect of OXT on DF_{GABA} in E18 and P2 neurons, before and after the birth-associated surge of endogenous OXT (Fig. 4). Moreover, no additive effect of bumetanide was observed in the presence of exogenous OXT, suggesting an occlusive interaction between OXT and bumetanide. Accordingly, bumetanide had little effect on DF_{GABA}, but reversed the effect of atosiban at term, suggesting that NKCC1 is down-regulated by endogenous OXT (Fig. 4).

How OXT inhibits NKCC1 is at present unknown. It might involve any of three modes of NKCC1 regulation, including suppression of NKCC1 synthesis, internalization from the cell membrane to cytoplasm and inhibition of NKCC1 activity via phosphorylation/dephosphorylation. There are arguments against a suppression of NKCC1 synthesis by OXT. Firstly, the expression levels of the mRNAs encoding for different determinants of intracellular chloride concentration were analysed using quantitative RT-PCR (Tyzio et al., 2006). These included cation chloride cotransporters (NKCC1, KCC1, KCC2, KCC3, KCC4), anion exchangers (AE3, NDBCE), the chloride channel ClC-2 and the kinase WNK3 known to regulate cation chloride cotransporter activity. However, no significant difference was found between mRNAs levels in the hippocampi of E21 rat pups delivered with caesarean section from control pregnant rats or rats receiving the OXT-R antagonist SSR126768A administered orally starting from E18. The second argument is based on the kinetics of the OXT effect. Using the intracellular fluorescent chloride sensor MQAE, it

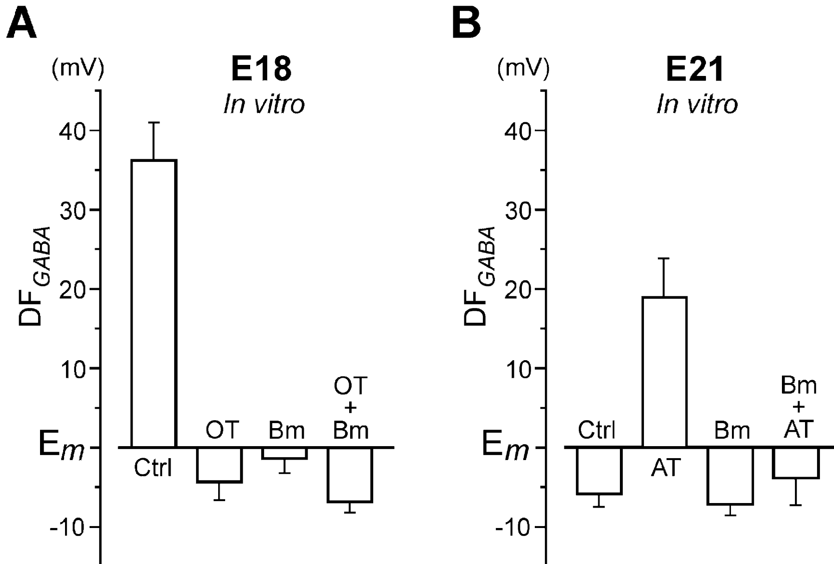


Fig. 4. Action of oxytocin on GABA signalling is mediated via down-regulation of the bumetanide-sensitive chloride transport. Histograms of DF_{GABA} measured in CA3 pyramidal cells using cell-attached recordings of single $GABA_A$ channels in E18 (A) and E21 (B) rat hippocampal slices. (A) At E18, bumetanide (Bm, 10 μM) mimics and occludes the depolarizing to hyperpolarizing switch in the GABA signalling mediated by exogenous OXT (OT, 1 μM). (B) At E21, bumetanide does not affect DF_{GABA} in control conditions when GABA is already hyperpolarizing. In the presence of atosiban (AT, 5 μM) to block the effects of endogenous oxytocin, bumetanide switches DF_{GABA} from depolarizing to hyperpolarizing. Pooled data from 45 cells (E18) and 50 cells (E21). Adapted with permission from Tyzio et al., 2006.

was shown that the OXT-induced reduction in $[Cl^-]_i$ develops within tens of seconds, too short to involve any modification in gene expression. Taken together, these two observations suggest that the OXT induced reduction in $[Cl^-]_i$ at birth does not involve a change in the expression of the molecules that control intracellular chloride homoeostasis. Clearly, further studies are required to establish the mechanisms linking the activation of OXT receptors and the molecules controlling chloride homoeostasis, NKCC1 being the most likely candidate.

Oxytocin as neuroprotector in perinatal hypoxia

Delivery is a stressful event associated with major risks to the foetus, and in particular to the foetal brain. Traumatic brain injury due to mechanical brain compression during passage via the delivery channel, and brain hypoxia/ischaemia due to displacement of placenta or umbilical strangulation/obstruction constitute the major causes of the intranatal death and poor neurological outcome (Volpe, 2000). Despite the clinical importance, the physiology and pathophysiology of the foetal brain during delivery are poorly understood. Important perinatal risks to the foetal brain raise the question of the mechanisms of adaptation of the foetal brain to birth, in which OXT may be involved. Various hypotheses regarding the role for OXT in the adaptation of the foetal brain to delivery can be put forward. For example, inhibition of brain activity may alleviate perception of the noxious stimuli present during delivery. So far, however only a role of OXT in preventing brain hypoxia has been demonstrated.

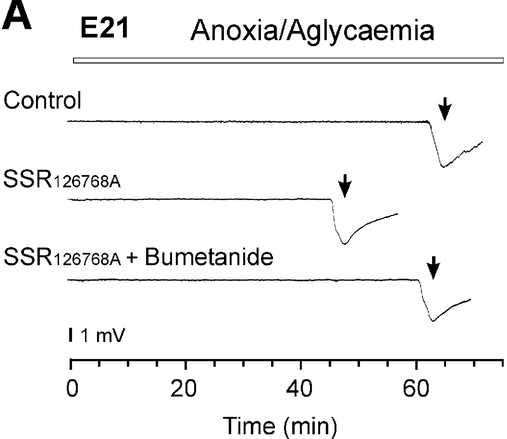
Glucose oxidation is the principal source of energy for the brain. Deprivation of oxygen and glucose during brain ischaemia results in a progressive decrease of intracellular energy metabolites and complex alterations in energy-dependent processes (Kass and Lipton, 1982; Katsura et al., 1993; for a review see, Hansen, 1985; Martin et al., 1994; Lipton, 1999). With regard to brain activity, two principal phases can be distinguished in the pathogenesis of brain ischaemia: a phase of 'adaptation' and a phase of anoxic depolarization (AD). During the adaptation phase, the brain switches to an energy-saving mode and the effects of anoxia are reversible. To save energy, neuronal activity is rapidly blocked and intracellular stores of energy are exclusively used to maintain the vital functions — to keep the ionic gradients and to maintain membrane potential. Studies of the mechanisms underlying this depression of neuronal activity during adaptation have emphasized the role of two factors: an adenosine-dependent reduction of glutamatergic transmission (Fowler, 1989; Zeng et al., 1992; Croning et al., 1995; Khazipov et al., 1995; Katchman and Hershkowitz, 1996; Dzhala et al., 1999, 2001) and neuronal hyperpolarization due to an activation of potassium conductances (Hansen et al., 1982; Fujiwara et al., 1987; Erdemli et al., 1998). The effects of anoxia during the adaptation phase are reversible, and re-oxygenation recovers brain function. The transition to the second phase, AD, occurs when the intracellular stores of energy metabolites become exhausted, and there is a rapid fall in the ATP concentration and a cessation of ATP-dependent ionic transporters including Na^+, K^+-ATPase. This results in major disturbances of the ionic gradients, including the accumulation of extracellular potassium, the entry of calcium and the activation of intracellular cascades leading to neuronal damage (Lipton, 1999; Lee et al., 1999; Muller and Somjen, 2000). As a result of ionic changes, neurons first depolarise gradually, then in an accelerated manner, and finally lose membrane potential. The late rapid phase of collective neuronal depolarization can be reflected as a negative shift of extracellular field potential — a rapid AD (Sick et al., 1987; Korf et al., 1988; Balestrino et al., 1989; Somjen et al., 1990; Lauritzen and Hansen, 1992; Katayama et al., 1992; Xie et al., 1995) that has many common features with the spreading depression phenomenon described by Leão (1947). If re-oxygenation is delayed for several minutes after AD, brain functions do not recover (Fairchild et al., 1988; Balestrino et al., 1989; Somjen et al., 1989; Tanaka et al., 1997; Dzhala et al., 2000). Therefore, AD can be used as an electrophysiological marker of neuronal death. The level of energy consumption during the adaptation phase is a critical factor determining the AD onset and

resistance to hypoxia. For example, boosting neuronal activity by blocking adenosine receptors or adding ictogenic agents strongly accelerates AD onset, while the suppression of seizures delays it (Dzhala et al., 2000). Because maintenance of elevated chloride in the immature neurons requires considerable amounts of energy, OXT-mediated inhibition of chloride loading should save energy during the adaptation phase thus increasing the brain resistance to hypoxia.

The intact hippocampal preparation was used to test this hypothesis (Tyzio et al., 2006). Episodes of anoxia/aglycaemia were induced by superfusion with a solution in which nitrogen was substituted for oxygen and sucrose was substituted for glucose. The onset of AD was measured using extracellular field potential recordings in the intact hippocampi of E21 rats in vitro (Fig. 5). It was found that (i) in control foetuses, AD occurs nearly 55 min after perfusion with the anoxic/aglycaemic solution; (ii) in the hippocampi prepared from foetuses intracardially perfused with OXT receptor antagonist atosiban or from the foetuses whose mothers received the OXT antagonist SSR126768A, AD onset was significantly accelerated to 44 min. To determine whether the reduction of intracellular chloride was responsible for the increased resistance to hypoxia, the effects of bumetanide were further tested. It was found that blockade of NKCC1 with bumetanide, applied in the presence of the OXTR antagonists (SSR126768A or atosiban) delayed AD to 53 min. These data indicate that maternal OXT exerts a neuroprotective action on foetal neurons during parturition probably via blockade of NKCC1 activity and thereby reduction in the brain metabolic demand (Tyzio et al., 2006).

Concluding remarks

Thus, OXT, in addition to its well established role in labour and lactation, and its multiple effects in the adult central nervous system (Argiolas and Gessa, 1991; Raggenbass, 2001; Gimpl and Fahrenholz, 2001; Tomizawa et al., 2003; Kosfeld et al., 2005;



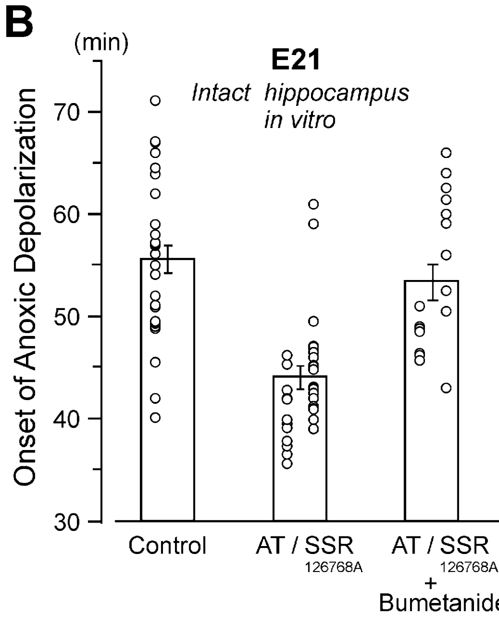


Fig. 5. Blockade of oxytocin receptors decreases foetal brain resistance to anoxia–aglycaemia at birth. (A) Extracellular field potential recordings from E21 intact hippocampi exposed to anoxic–aglycaemic solution. Note terminal anoxic depolarization that marks neuronal death (arrows). Anoxic depolarization occurs earlier in the presence of the OXT receptor antagonist SSR126768A and it is delayed by addition of NKCC1 antagonist bumetanide (10 μM). (B) Summary plot of the onset of anoxic depolarization in control, in the presence of the OXT receptors antagonists atosiban (5 μM, foetal intracardial perfusion) and SSR126768A (1 mg/kg to the mother), and after further addition of bumetanide (10 μM). Each circle corresponds to one hippocampus ($n = 82$ intact hippocampi; E21). Adapted with permission from Tyzio et al., 2006.

Huber et al., 2005; Theodosis et al., 2006), also exerts a powerful action on foetal neurons. The hormone produces a switch in the action of GABA from depolarizing to hyperpolarizing and augments the resistance of foetal brain to anoxia. The dual action produced by a single messenger in the mother and foetus enables a perfect timing for adaptation of foetal neurons to delivery. In further research, it will be important to determine the transduction mechanism linking the OXT receptor with the chloride homoeostasis system. The most likely mechanism involves inhibition of NKCC1 activity via phosphorylation/dephosphorylation or internalization, but this hypothesis remains to be verified. The OXT-mediated switch in the GABA actions should have a major impact on the activity of neuronal networks and the effects of perinatal OXT on the patterned activities in the brain remain to be elucidated. In particular, it would be of interest to determine the effects of OXT on the giant depolarizing potentials, which are dependent on excitatory GABA. The perinatal GABA switch also raises a hypothesis that OXT may modulate paroxysmal activities dependent on excitatory GABA. This could explain the fact that non-symptomatic neonatal seizures often start at about the end of the first postnatal day. Such a delay in the seizure occurrence could reflect a relief from the OXT-mediated inhibition at birth. Important questions remain about the neuroprotective effects of OXT. So far the phenomenon of neuroprotection has been only demonstrated in vitro, and it remains to be proven in the animal models in vivo, or in human neonates. It would also be important to explore whether OXT protects the brain from other adverse factors associated with the delivery. Finally, because delivery is associated with significant changes not only in OXT but also other hormones, it would be important to determine how OXT works in the orchestration of other systems adapting the foetal brain to delivery.

Abbreviations

AD	anoxic depolarization
DF_{GABA}	$GABA_A$ driving force
E	embryonic day
E_{GABA}	$GABA_A$ reversal potential
E_m	resting membrane potential
GABA	gamma-aminobutyric acid
MQAE	*N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide
NMDA	*N*-methyl-D-aspartate
OXT	oxytocin
P	postnatal day
PCR	polymerase chain reaction

Acknowledgements

We thank our colleagues Drs. R. Cossart, I. Khalilov, M. Minlebaev, A. Represa and C. Hubner for their contribution to the original experimental studies. This work was supported by INSERM, Fondation pour la Recherche Médicale, L'Agence Nationale de la Recherche, Federation pour la Recherche sur le Cerveau and Rotary International, Conseil régional Provence-Alpes-Côte d'Azur and European Community.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 22

Central vasopressin and oxytocin release: regulation of complex social behaviours

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Abstract: The neuropeptides arginine vasopressin (AVP) and oxytocin (OXT) are acknowledged as important modulators of diverse social behaviours. Here we discuss recent studies using intracerebral microdialysis to investigate the dynamics of AVP and OXT release patterns within distinct brain regions during the display of social behaviours in rats. Manipulation of local receptor-mediated actions of AVP and OXT via retrodialysis of either agonists or antagonists revealed the behavioural significance of changes in local neuropeptide release. Alterations in local AVP and OXT within, e.g. the medio-lateral septum, the central amygdala or the hypothalamic paraventricular nucleus (PVN) were associated with intermale and maternal aggression, respectively. Moreover, increased OXT release within the PVN was associated with male sexual behaviour and successful mating. Using retrodialysis, we found that AVP released within the lateral septum during the resident–intruder test was associated with anxiety-related behaviour and with non-aggressive social behaviour rather than intermale aggressive behaviour. In contrast, OXT release within the PVN and the central amygdala correlated positively with the level of maternal aggression. Interestingly, OXT released within the PVN during sexual activity in male rats was found to be associated with a robust decrease in anxiety-related behaviour up to 4 h after mating. These data illustrate distinct modes of behavioural actions of AVP and OXT, reaching from acute regulation of the respective social behaviour to the long-term modulation of related behaviours including anxiety and social cognition. In conclusion, measuring the in vivo release patterns of AVP and OXT within distinct brain regions during the display of diverse social behaviours and manipulation of local AVP and OXT activity has yielded new insights into the specific roles of these neuropeptides in the regulation of complex social behaviours.

Keywords: amygdala; anxiety; hypothalamus; male aggression; maternal aggression; microdialysis; septum; sexual behaviour

Introduction

The neuropeptides arginine vasopressin (AVP) and oxytocin (OXT) are closely related, highly conserved neuropeptides that are synthesized in the paraventricular (PVN) and supraoptic (SON) nuclei (Buijs et al., 1983; Landgraf and Neumann, 2004). Additionally, AVP synthesizing neurons have been found in the suprachiasmatic nucleus, the medial amygdala and the bed nucleus of the stria terminalis (BNST) (De Vries and Buijs, 1983). However, synthesis of AVP and/or OXT in further brain regions might be possible (Planas et al., 1995;

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DOI: 10.1016/S0079-6123(08)00422-6

Chodobski et al., 1998; Hallbeck et al., 1999). Due to the wide-spread distribution of AVP V1a receptors (V1aR), AVP V1b receptors (V1bR) and OXT receptors (OXTR) within the brain, central AVP and OXT can alter the neuronal activity within many brain regions, including the lateral septum, central amygdala, hippocampus, hypothalamic and brain stem regions (Barberis and Tribollet, 1996; Hernando et al., 2001; Huber et al., 2005).

AVP and OXT, and their respective ancestral peptides arginine vasotocin (AVT) and mesotocin, are established as regulators of various social behaviours across species, including rats, mice, hamsters, voles, birds and fish (Ferris, 1992; Engelmann et al., 2000; Goodson and Bass, 2001; Storm and Tecott, 2005; De Vries and Panzica, 2006). The locations of the respective neuropeptidergic neurons and fibres within the brain have been strongly conserved during vertebrate evolution (Moore and Lowry, 1998; Goodson and Bass, 2001). Moreover, the brain circuitries that regulate basic forms of social behaviour are strikingly similar across different vertebrate species (Newman, 1999; Goodson, 2005). This so-called 'social behaviour network' comprises core brain structures including the medial amygdala, BNST, lateral septum, medial preoptic area, the anterior hypothalamus, the ventromedial hypothalamus and the periaqueductal grey (Newman, 1999; Goodson, 2005). Interestingly, AVP and OXT and/or their receptors are expressed in most of these regions, strongly suggesting their role as integral components of the social behaviour network. Among other factors, differences in neuropeptide synthesis, neuropeptide release and neuropeptide receptor distribution within the social behaviour network probably provide the basis for the diversity of social organizations found among vertebrate species, between males and females and even among individuals (Insel and Shapiro, 1992; Insel et al., 1994; Wang et al., 1994; Young et al., 1997; Bester-Meredith et al., 1999; D'Eath et al., 2005; Veenema et al., 2006, 2007a).

An additional and important approach in understanding the role of the brain AVP and OXT systems in the regulation of complex social behaviours is to monitor the release patterns of endogenous AVP and OXT within distinct brain regions during the display of social behaviours using intracerebral microdialysis. Such an approach is especially important, as neuropeptide concentrations in blood plasma do not reflect the dynamics of intracerebral, locally restricted release. Moreover, to reveal whether changes in local release of AVP and OXT are causally involved in the display of social behaviours, the AVP or OXT system can be locally manipulated via application of neuropeptide receptor agonists or antagonists using retrodialysis and simultaneous monitoring of behavioural consequences. Over the last few years, we have begun to unravel the dynamics and behavioural consequences of endogenous AVP and OXT release in the context of social behaviours including intermale aggression, maternal aggression and mating behaviour (see Table 1 and Fig. 1).

Monitoring of local release of AVP and OXT during social behaviour by intracerebral microdialysis

Intracerebral microdialysis is an in vivo technique, which enables the monitoring of local extracellular concentrations of neuropeptides (and other substances like amino acids and catecholamines) within restricted brain regions. This technique therefore provides unique information on the potential signal function of the neurotransmitter or neuromodulator of interest. Microdialysis is based on the principle that substances in the local extracellular fluid will diffuse from a higher to a lower concentration, i.e. into the dialysing medium through the semi-permeable membrane of the microdialysis probe (molecular cutoff of 18 kDa) inserted into the selected brain region. Regardless of its limitations, intracerebral microdialysis has been successfully used to measure changes in local extracellular concentrations of a particular substance in response to pharmacological, stressful or social stimuli (Westerink, 1995; Horn and Engelmann, 2001; Landgraf and Neumann, 2004). Despite the large number of microdialysis studies and its routine methodology, the use of microdialysis during the display of various social behaviours is still challenging (Table 1). Therefore, we will address a few methodological aspects based on

Table 1. Social stimuli inducing dynamic changes in local AVP and/or OXT release within the given brain region

	Brain region	AVP	OXT	References
Parturition				
Sheep	OB	=	↑	Kendrick et al. (1988), Levy et al. (1995)
Rat	SON	=	↑	Neumann et al. (1993b)
Rat	PVN	=	↑	Neumann et al. (1993b)
Rat	Septum	=	=	Landgraf et al. (1991)
Rat	Hippocampus	↑	=	Landgraf et al. (1991)
Sheep	Substantia nigra	n.m.	↑	Kendrick et al. (1988)
Suckling				
Sheep	OB	n.m.	↑	Kendrick et al. (1988)
Rat	SON	=	↑	Moos et al. (1989), Neumann et al. (1993a, b)
Rat	PVN	=	↑	Bealer and Crowley (2001), Neumann et al. (1993b)
Sheep	Substantia nigra	n.m.	↑	Kendrick et al. (1988)
Aggression				
Female rats	Septum (NAB)	n.m.	=	Bosch et al. (2004)
	PVN (NAB, HAB)	n.m.	↑	Bosch et al. (2004, 2005)
	PVN (LAB)	n.m.	↓	Bosch et al. (2004, 2005)
	CeA (NAB, HAB)	n.m.	↑	Bosch et al. (2004, 2005)
Male rats	Septum (LAB)	↓	=	Beiderbeck et al. (2007)
	Septum (NAB,[a] HAB)	↑	=	Beiderbeck et al. (2007)
Social defeat				
Female rats	Septum	n.m.	=	Bosch et al. (2004)
	PVN	n.m.	↑	Bosch et al. (2004)
	CeA	n.m.	=	Bosch et al. (2004)
Male rats	Septum	=	↑	Ebner et al. (2000)
	SON	n.m.	↑	Engelmann et al. (1999)
	PVN	↑	=	Wotjak et al. (1996)
Mating				
Male rats	PVN	n.m.	↑	Waldherr and Neumann (2007)

Notes: Aggressive behaviour was measured in resident rats exposed in their home cage to an unknown intruder for 10 min. OB, olfactory bulbus; SON, supraoptic nucleus; PVN, paraventricular nucleus; CeA, central amygdala; NAB, non-selected Wistar rat; HAB, Wistar rat genetically selected for high-anxiety-related behaviour on the elevated plus maze; LAB, Wistar rat genetically selected for low-anxiety-related behaviour on the elevated plus-maze; ↑ increase in neuropeptide release during exposure to the social stimulus compared with basal neuropeptide release; = no change in local neuropeptide release; n.m., not measured.

[a]Veenema and Neumann (unpublished data); see text for details.

our experience of monitoring AVP and OXT release during social behaviours in rats.

A first prerequisite is the free interaction between the experimental and the stimulus animal. This requires the solid fixation of the microdialysis probe within the target brain region. Therefore, we use U-shaped microdialysis probes, which are chronically implanted (Neumann et al., 1993b). Chronic implantation causes the growth of a glia sheet around the microdialysis membrane, which restricts the time window for subsequent microdialysis studies to 2–3 days after probe implantation (Landgraf, 1995; Horn and Engelmann, 2001). Accordingly, the postoperative recovery period is relatively short (2–3 days). Therefore, careful behavioural observations and comparisons with non-manipulated animals are required to ensure that the experimental animal displays normal social behaviour. In our hands, recent surgery and ongoing microdialysis does not affect the behavioural performance during parturition, suckling, maternal care, maternal aggression, intermale aggression, or sexual behaviour (Neumann et al., 1993b; Bosch et al., 2004, 2005; Beiderbeck et al., 2007; Waldherr and Neumann, 2007). Second, the number of samples to be

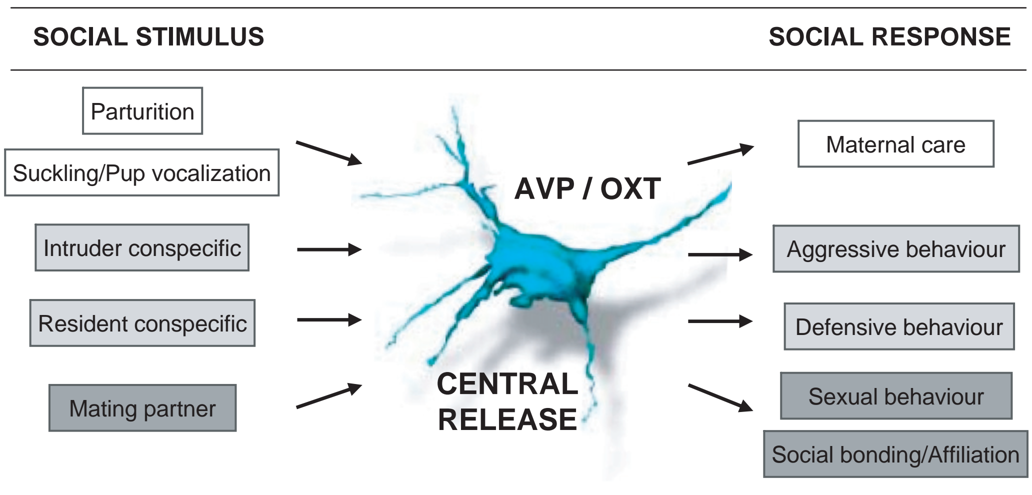

Fig. 1. Modulation of social behaviours by the neuropeptides AVP and OXT upon their release within distinct brain regions in response to various social stimuli. The type of social behaviour the animal will show depends on the social context and on the type of social stimulus. The latter requires the ability to identify and recognize a conspecific. AVP and OXT were shown to be crucially involved in social recognition (reviewed in Bielsky and Young, 2004).

collected, the sampling interval and the flow rate largely depend on the design of the experiment and on the expected outcome. We typically collect the first two samples under basal (undisturbed) conditions, followed by 1–2 samples including the exposure to the defined social stimulus and the last two samples are again taken under undisturbed conditions. For monitoring neuropeptide release we generally use a sampling interval of 30 min and a flow rate of 3.3 μL/min (Neumann et al., 1993b; Bosch et al., 2004, 2005; Beiderbeck et al., 2007). Shorter sampling intervals and higher flow rates might be considered, if short-lasting changes in extracellular neuropeptide concentrations are expected (Waldherr and Neumann, 2007). However, the sensitivity of the analytical method should be taken into consideration. Third, the behavioural relevance of changes in local neuropeptide release can be ensured (1) by comparing the neuropeptide content in samples collected inside and outside the respective target area (Bosch et al., 2005; Waldherr and Neumann, 2007), (2) by comparing OXT with AVP release patterns, (3) by comparing neuropeptide release patterns between animals which show extremes in the respective behavioural phenotype (Bosch et al., 2005; Beiderbeck et al., 2007) or (4) by comparing neuropeptide release patterns in the same animal exposed to a social and a non-social (i.e. forced swim) test on 2 consecutive days. Finally, although almost any brain region can be targeted for microdialysis purposes, we have to keep in mind that, in case of the hypothalamic PVN, we cannot distinguish between local release from either parvo- or magnocellular neuropeptide neurons with different projection targets like the central amygdala or the neurohypophysis, respectively. The precise sites of neuropeptide release might reflect the activity of different neuronal populations and, thus, might have functional implications for the regulation of autonomic, behavioural or neuroendocrine functions.

Importantly, the microdialysis technique can be used to locally administer a selective receptor agonist or antagonist during ongoing behavioural testing, thus mimicking changes in local release and in subsequent neuropeptide receptor interactions, respectively. Reversed microdialysis (or retrodialysis) is an elegant method to demonstrate the behavioural relevance of the previously observed change in local neuropeptide release during the display of a certain type of social behaviour (see below).

Behavioural relevance of local neuropeptide release within target brain regions

Intracerebral AVP and OXT release during the display of male aggressive behaviour

Human and animal studies suggest an important role for AVP and OXT in the regulation of male social behaviours, especially intermale aggression (Koolhaas et al., 1990; Albers and Bamshad, 1998; Coccaro et al., 1998; Ferris, 2005). Any type of social interaction requires the communication between conspecifics, in which they use species-specific social and behavioural cues to be able to 'understand' and 'read' the intentions of the other, known as 'theory of mind'. Interestingly, intranasal AVP administration in humans decreased the perception of friendly faces and increased the perception of anger and threat to neutral human facial expressions (Thompson et al., 2004, 2006). In contrast, intranasal OT application improved the ability to 'read' the mental state of others from facial cues and promotes pro-social approach behaviours (Kosfeld et al., 2005; Domes et al., 2007). These studies suggest that AVP and OXT play important, albeit different, roles in social communication. Hence, abnormalities in the AVP and/or OXT system might significantly influence male aggressive and other social behaviours and might contribute to social behavioural dysfunctions. Indeed, excessive aggression in personality-disordered subjects was found to correlate with high AVP concentrations in the cerebrospinal fluid (Coccaro et al., 1998), which likely reflects — at least globally — central release patterns (Landgraf and Neumann, 2004).

Although these findings suggest a simple positive correlation between AVP and aggression, extensive studies in several species demonstrate that the role of AVP/AVT in the regulation of aggression is rather complex and equivocal. The AVP circuit involved in male aggressive and territorial behaviours comprises AVP neurons originating in the medial amygdala and the BNST and projecting to the lateral septum (De Vries and Buijs, 1983; Koolhaas et al., 1998), and of AVP projections to hypothalamic regions, especially the anterior hypothalamus (Ferris et al., 1997; Gobrogge et al., 2007). With respect to the ancestral peptide AVT, similar central pathways were described to be involved in male aggressive behaviours of, e.g. birds (Goodson, 1998a; Goodson and Adkins-Regan, 1999; Goodson and Bass, 2001). Pharmacological (administration of synthetic AVP/AVT or specific V1aR/V1bR antagonists), anatomical (immunocytochemistry, receptor autoradiography) and genetic (knockout mice, selection lines, species comparisons) studies have indicated that variations in aggression, which are inherent to general variations in social organization, are likely to be functionally associated with differences in the anatomical organization and sensitivity of the AVP or AVT systems. These findings provide a rationale for understanding the variability in the effects of AVP or AVT on male aggressive behaviours. For example, opposite effects of AVT were observed in territorial versus colonial male songbirds, where AVT infused into the septum inhibits aggression in territorial field sparrow and violet-eared waxbill, but facilitates aggression in the colonial zebra finch (Goodson, 1998a, b; Goodson and Adkins-Regan, 1999). Moreover, centrally administered AVP promotes mating-induced pair bonding and selective aggression in monogamous male prairie voles, but not in polygamous male montane voles (Young et al., 1997, 1999). Yet, enhancing V1aR expression in the ventral forebrain by viral vector-mediated gene-transfer induced pair-bond formation in otherwise polygamous male meadow voles (Lim et al., 2004). Although it is unknown whether this enhanced V1aR expression also promotes aggression in otherwise low-aggressive male meadow voles, these data clearly suggest that structural differences within the AVP system can be correlated with functional differences in social behaviour, like intermale aggression.

However, there is in general limited information on the central release of AVP, e.g. within the septum, during intermale aggression. The septal area is of particular interest, as it receives a high input of AVP fibres and has an abundant expression of V1aR in rats (De Vries and Buijs, 1983; Ostrowski et al., 1992). Furthermore, AVP within the septum has been implicated in the regulation of aggression, although its precise role

is still unclear (Koolhaas et al., 1990; Ferris, 2005). For example, acute infusion of AVP into the lateral septum increased aggressive behaviours in castrated male rats (Koolhaas et al., 1991) and in male golden hamsters (Ferris and Delville, 1994). However, as the septum of castrated rats and golden hamsters is almost devoid of AVP-immunoreactive fibres (DeVries et al., 1985; Ferris and Delville, 1994; Ferris et al., 1995), the functional implication of these studies remains obscure. Studies with two *Peromyscus* species suggest a positive correlation between septal V1aR and intermale aggression (Bester-Meredith et al., 1999). However, immunocytochemical studies in wild-type rats and in genetically selected wild house mice rather suggest a negative correlation between septal AVP and intermale aggression (Compaan et al., 1993; Everts et al., 1997).

We used male Wistar rats bred for low (LAB) or high (HAB) anxiety-related behaviour (Landgraf et al., 2007) to monitor AVP release within the septum during intermale aggression. These rat breeding lines show a clear difference in intermale aggression. LAB rats have a shorter attack latency and display a higher level of aggressive behaviour than HAB rats when they are exposed to an unknown male conspecific in their home-cage (Beiderbeck et al., 2007; Veenema et al., 2007b). Moreover, LAB rats show a lower level of non-aggressive social interactions. Using intracerebral microdialysis, we could demonstrate dynamic changes in septal AVP release during the display of intermale aggression. Thus, a high level of aggressive behaviour as found in LAB resident males was associated with a significant decrease in septal AVP release (Beiderbeck et al., 2007). In contrast, a low level of aggression of HAB resident males was accompanied by an increase in septal AVP release. These findings favour a negative correlation between septal AVP and intermale aggression in these rat lines.

To reveal the behavioural relevance of these opposing AVP release patterns within the septum, high-aggressive LAB rats were locally treated with synthetic AVP and low-aggressive HAB rats with the specific V1aR antagonist $d(CH_2)_5Tyr(Me)AVP$ (kindly provided by Dr. Maurice Manning, Toledo, Ohio) applied into the septum via retrodialysis. In contrast to our expectations, manipulation of septal AVP activity did not alter the level of aggression in either LAB or HAB males (Beiderbeck et al., 2007; see Fig. 2). However, local treatment with the specific V1aR antagonist reduced the relatively high level of non-aggressive social interactions in HAB rats to the level seen in vehicle-treated LAB rats. Moreover, subsequent exposure to the elevated plus-maze revealed that local AVP application induced a significant increase in anxiety-related behaviour of LAB rats (Beiderbeck et al., 2007). These results emphasize the diverse behavioural functions of AVP within the septum regulating social cognition, anxiety and stress coping (reviewed in Engelmann et al., 1996). With this in mind, we propose an additional mechanism of action for septal AVP. A dynamic change in septal AVP release could be the consequence, rather than the cause, of the display of aggression. In this way, aggressive behaviour elicits a change in septal AVP release, which, in turn, affects other closely related behaviours, including social cognition and anxiety (Fig. 3). Thus, aggression-induced changes in septal AVP release might alter other behaviours, which are beneficial and relevant in the context of aggression. This might, in general, implicate that AVP is essential for the functional integration of diverse aspects of behaviours including aggression, anxiety and social cognition, a hypothesis that will be tested in future studies.

As the LAB and HAB rat lines have been selected for a genetic difference in anxiety over more than a decade, and LAB rats display higher levels of aggression compared with non-selected Wistar rats (normal anxiety-related behaviour (NAB) rats, purchased from Charles River, Germany; Veenema et al., 2007b), it is possible that the septal AVP release patterns are unique to these rat lines. Therefore, we also measured the in vivo release of AVP within the septum of NAB residents during the resident–intruder test. Based on the behavioural scoring, male rats were divided into aggressive (one or more attacks) and non-aggressive (no attacks) rats. Interestingly, a significant increase in AVP release was found in aggressive NAB resident rats, whereas it remained unchanged in non-aggressive NAB residents compared with

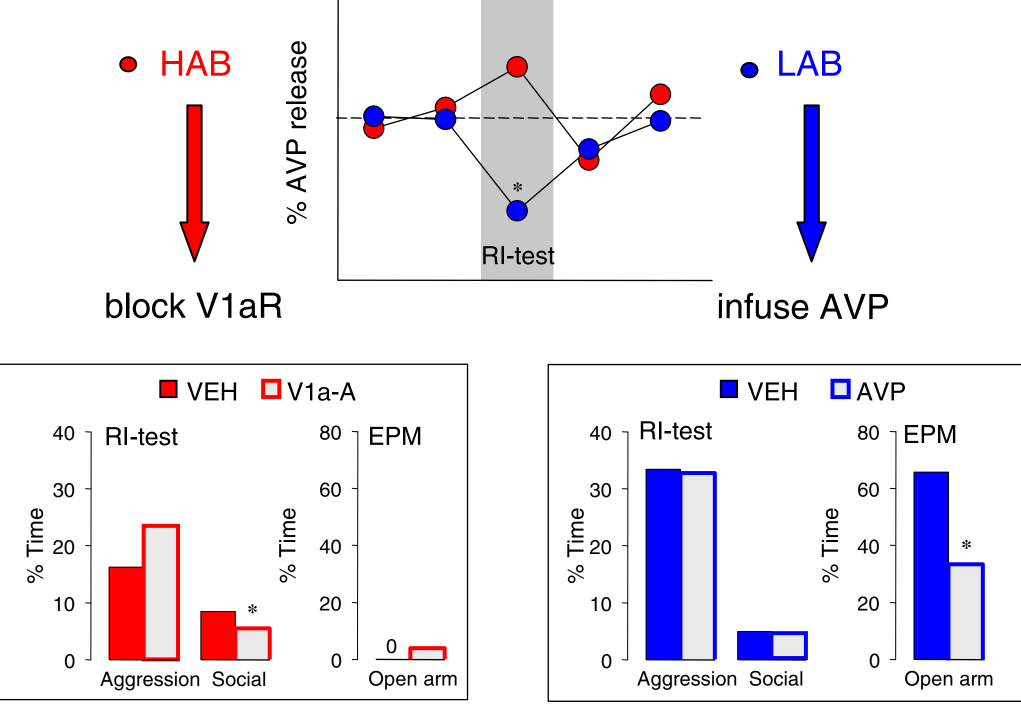

Fig. 2. Based on opposing AVP release patterns within the septum of low-aggressive HAB and high-aggressive LAB resident male rats exposed to the resident–intruder (RI) test, a specific V1aR antagonist (V1a-A; 10 μg/mL) was applied into the septum of HAB males, while LAB males were locally given synthetic AVP (1 μg/mL) into the septum via retrodialysis (flow rate 3.3 μL/min). Thirty minutes after the beginning of the retrodialysis procedure, HAB and LAB residents were tested for aggression during the 10-min RI-test. Twenty minutes later, the microdialysis probes were disconnected and the rats were tested for anxiety-related behaviour on the elevated plus-maze (EPM). Manipulation of septal AVP activity had no effect on aggression in either rat line (despite a non-significant tendency in HAB rats), but reduced social investigation in HAB rats treated with the V1a-A, and increased anxiety-related behaviour (reduction in percentage time spent on open arms) in LAB rats treated with synthetic AVP. $^{*}p<0.05$ versus HAB (release) or versus VEH (behaviour). Adapted with permission from Beiderbeck et al., 2007. (See Color Plate 22.2 in color plate section.)

basal AVP release (Veenema and Neumann, unpublished observation). The behavioural significance of the aggression-induced increase in septal AVP release is still awaiting. These findings however signify the importance of including more strains or species in these microdialysis studies.

There are several indications for a role of brain OXT in social behaviours in men, including initial studies demonstrating a link between autism spectrum disorders and polymorphisms in the OXTR gene and changes in OXT availability (Green et al., 2001; Hollander et al., 2003; Wu et al., 2005). Social memory deficits have been found in mice lacking either OXT or the OXTR, while effects on intermale aggression seem ambiguous (DeVries et al., 1997; Ferguson et al., 2000; Winslow and Insel, 2002; Takayanagi et al., 2005). It would therefore be of interest to investigate local release patterns of OXT during the display of male aggressive behaviour.

Intracerebral OXT release during the display of female social behaviours

Physiological and behavioural changes have been extensively described in the mammalian maternal brain occurring in the peripartum period (Neumann, 2001). These profound adaptations start during pregnancy as complex and direct

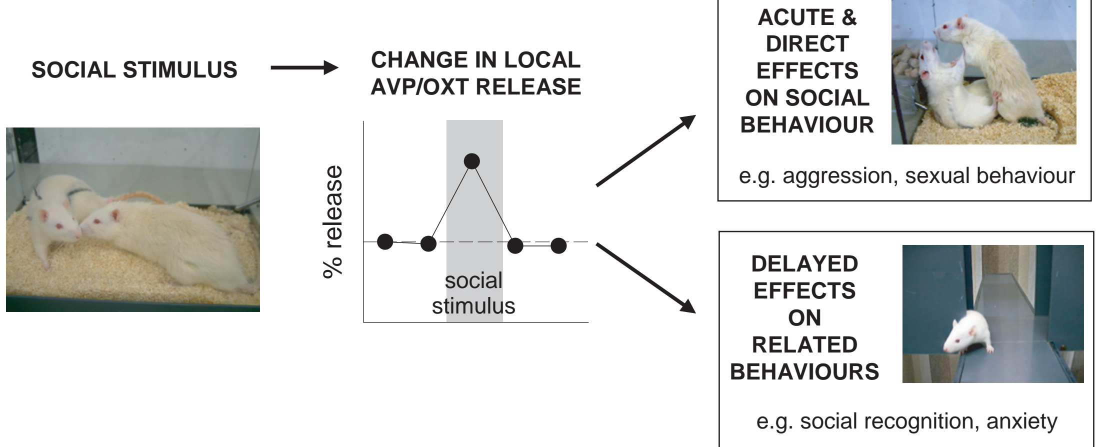

Fig. 3. Hypothetical model of two distinct modes of action for the neuropeptides AVP and OXT. A social stimulus-induced change in local neuropeptide release: (1) can have an acute and direct behavioural effect during the presence of the social stimulus, e.g. the facilitation of aggressive behaviour displayed by a resident rat towards an intruder rat and (2) can modify other types of behaviour, which are relevant in the context of the social stimulus, such as the acquisition of social memory or a change in anxiety-related behaviour. See text for further explanations.

consequences of mainly hormonal signals arising from the foetus. They continue around birth and in lactation as a result of close social interactions between mother and offspring, e.g. during suckling, maternal care and protection.

The OXT system plays a predominant role in female reproduction. The neurohypophysial neurohormone OXT promotes uterine contractions during labour thus contributing to a rapid and safe delivery process, and triggers milk ejection in the lactating mammal. In addition, the brain OXT system becomes activated as witnessed by increased OXT synthesis, local OXT release and OXTR expression and binding in several brain regions (Neumann et al., 1993b; Insel et al., 1997; Pedersen, 1997; Numan and Insel, 2003; Meddle et al., 2007). For example, it has been demonstrated in rats and sheep that OXT release occurs within selected brain regions during parturition and in response to the suckling stimulus in the lactating animal (Table 1). Using intracerebral microdialysis or push–pull perfusion techniques, such local release of OXT has been shown, e.g. within the hypothalamic SON, PVN and the olfactory bulb during parturition (Kendrick et al., 1988; Neumann et al., 1993b). In addition to these brain regions (Kendrick et al., 1988; Moos et al., 1989; Neumann et al., 1993b), OXT release was also demonstrated within the hippocampus and the medio-lateral septum in response to somatosensory stimulation provided by the suckling pups (Neumann and Landgraf, 1989). Such centrally released OXT acts in synergism with OXT secreted into peripheral circulation, thus regulating fine-tuned neuroendocrine functions (Moos et al., 1984; Ingram and Moos, 1992; Neumann et al., 1994, 1996) as well as close social interactions with the offspring including maternal behaviour (different nursing postures, pup retrieval in rats) and offspring recognition (sheep) (Kendrick et al., 1988; Pedersen, 1997). There is also emerging evidence for central OXT modulating maternal aggression necessary for the protection of the offspring (Erskine et al., 1978; Consiglio and Lucion, 1996; Elliott et al., 2001; Lubin et al., 2003). Protection of the offspring and enhanced aggressive behaviour towards potential conspecific encounters is part of the complex pattern of maternal behaviour in mammals (Numan and Insel, 2003).

Recently, we have quantified the release of OXT within the hypothalamic PVN and the central amygdala of lactating rat dams during the maternal defence test, i.e. the defence of the

pups against a virgin intruder in the home cage of the dam. Intracerebral microdialysis allowed simultaneous monitoring of locally restricted release patterns as well as of various maternal aggressive and non-aggressive aspects of social behaviour of the dam. In order to correlate the behavioural performance with the dynamics of OXT release within the PVN and the central amygdala, we used the genetically selected HAB and LAB rat lines. HAB and LAB dams differ not only in their level of anxiety, but also in the performance of maternal behaviour: HAB dams show a higher level of maternal care and pup retrieval and more maternal aggression towards a virgin intruder compared with LAB dams (Bosch et al., 2005; Neumann et al., 2005). A significant increase in OXT release within the PVN and the central amygdala was detected in high-aggressive HAB dams (Bosch et al., 2005). In contrast, in low-aggressive LAB dams, local release of OXT within the PVN was even found to decrease during the presence of the virgin intruder, while OXT release within the central amygdala was only slightly increased (Bosch et al., 2005). In both of these limbic brain regions, OXT release was directly correlated with the intensity of maternal aggression, indicating a direct link between local neuropeptide release and behavioural performance (Bosch et al., 2005).

To reveal the behavioural relevance of locally released OXT, we manipulated the ligand–receptor interactions within the brain target region during behavioural testing by retrodialysis. HAB dams treated bilaterally with the OXT receptor antagonist des-Gly-NH_2,d$(CH_2)_5$[Tyr(Me)^2,-Thr^4]OVT (kindly provided by Dr. Maurice Manning, Toledo, Ohio) within the PVN and within the central amygdala displayed a significant reduction in aggressive behaviour towards the virgin intruder (Bosch et al., 2005). Neither the level of pup-directed maternal behaviour nor the level of exploration during the 10-min maternal defence test was altered by the treatment. As in LAB dams, the release of OXT within the PVN was found to be reduced during the maternal defence test, we applied synthetic OXT via bilateral retrodialysis into the left and right PVN. We found a trend towards increased maternal aggression, i.e. OXT-infused LAB dams tended to display more lateral threats and offensive behaviour towards the virgin intruder (Bosch et al., 2005). These experiments provide evidence for a functional role of brain OXT locally released within the hypothalamus and the amygdala in the regulation of maternal aggression in the peripartum period. In these brain regions, OXT has been shown to exert direct regulatory effects on neuronal activity (Huber et al., 2005; Blume et al., 2008) and exerts anxiolytic effects in female and male rats (Bale et al., 2001; Neumann, 2002; Blume et al., 2008). Therefore, it is tempting to suggest a functional link between locally released OXT in response to a social challenge, i.e. exposure to the intruder, the reduction in anxiety and the display of maternal aggressive behaviour (Fig. 3).

Microdialysis perfusions were also performed in virgin intruder rats exposed to lactating residents during the maternal defence test. In intruders, an increased release of OXT was found in the PVN, but neither in the central amygdala nor in the lateral septum (Bosch et al., 2004). Increased OXT release within the PVN has also been found in response to a variety of other stressors including forced swimming, psychosocial and pharmacological stressors in both male and female rats (for review, see Landgraf and Neumann, 2004; Neumann, 2007), and is therefore likely to be related with the regulation of neuroendocrine stress responses (Neumann et al., 2000).

In conclusion, the high activity of the brain OXT system in the peripartum period is importantly involved in various adaptations of social behaviour including pup-directed behaviour and the display of relevant aggressive behaviours necessary for the protection of the offspring. Individual differences in local release patterns of OXT rather than differences in regional OXTR expression or binding (Bosch et al., 2005) seem to determine differences in these complex social behaviours in the lactating animal.

Compared with OXT, there is very limited evidence so far on the role of brain AVP in the regulation of maternal aggression (Nephew and Bridges, 2007).

Intracerebral OXT release during the display of sexual behaviour

As in females (Wigger and Neumann, 2002; Bosch et al., 2005), OXT is released within several regions of the male brain, including the hypothalamic PVN, in response to social defeat (Wotjak et al., 1996; Ebner et al., 2000) and non-social stressors (Hattori et al., 1992; Wotjak et al., 2001; Ebner et al., 2005; for review, see Landgraf and Neumann, 2004). However, in general, there is limited evidence for the activation of the brain OXT system during social interaction in males, despite the recent description of several pro-social effects of OXT in men after intranasal application (Heinrichs et al., 2003; Kirsch et al., 2005; Kosfeld et al., 2005; Domes et al., 2007).

Sexual interaction is the most intense social interaction found in males. Therefore, mating behaviour is likely to be a relevant stimulus for intracerebral OXT release. In support, increased Fos expression was found in OXT neurons within the PVN in response to mating, suggesting an increased activity of OXT neurons (Flanagan et al., 1993; Witt and Insel, 1994). Moreover, brain OXT plays an important role in the regulation of male sexual behaviour (Argiolas and Gessa, 1991). Activation of the OXT system in general is also reflected by an increased OXT secretion into blood during sexual behaviour and mating (Stoneham et al., 1985; Carmichael et al., 1987). Furthermore, intracerebroventricular (i.c.v.) application of an OXTR antagonist blocked the mating-induced pair bonding in monogamous female prairie voles, strongly suggesting release of OXT within relevant brain regions during mating (Insel and Hulihan, 1995).

We recently demonstrated that intracerebral OXT release during mating is not limited to monogamous mammals as it also occurs in polygamous rats. Using intracerebral microdialysis performed in freely moving male rats, we monitored OXT release within the PVN, a brain region importantly involved in the regulation of male sexual behaviour, stress coping, and anxiety (Li et al., 1996; Herman and Cullinan, 1997; Neumann, 2002; Argiolas and Melis, 2004; Blume et al., 2008). Fifteen-minute samples were collected within the PVN during single-housing, in the presence of an oestrogen-primed female behind a perforated wall, and after removal of the wall allowing physical contact and sexual behaviour (Waldherr and Neumann, 2007). Interestingly, local OXT release already started to rise during the presence of the primed female behind the wall, which allowed olfactory and visual, but not physical, contact or mating. As males clearly displayed signs of behavioural arousal under these conditions, OXT activation may already be induced by the presence of a receptive female even without mating, although this effect was not found to be significant. However, during the mating period, a significant rise in the release of OXT was found within the PVN, which declined after removal of the female from the male's cage (Waldherr and Neumann, 2007). This locally released OXT during mating is likely to be involved in a broad variety of complex behaviours including the regulation of male sexual behaviour (Argiolas and Melis, 2004), but also of mating-induced activation of several stress systems including autonomic and hypothalamic-pituitary-adrenal axis responses.

We therefore hypothesized that the release of OXT within the brain has far-reaching behavioural consequences and beneficial effects for the male rat, i.e. reducing the level of anxiety and increasing risk-taking behaviour for several hours. Indeed, when exposing mated and non-mated males to the elevated plus-maze and light-dark box, a clear reduction in anxiety-related behaviour was found up to 4 h after mating (Waldherr and Neumann, 2007). This anxiolytic effect of sexual activity could be blocked by an OXTR antagonist administered i.c.v. immediately after mating. These results provide evidence that the activated brain OXT system, as a consequence of sexual activity, mediates the mating-induced anxiolytic effect. The mating-induced anxiolytic effect might enable an advantageous behavioural strategy for a polygamous male rodent, promoting the unsafe search for novel mating partners with the ultimate goal to optimize the distribution of its genes. In humans, there is anecdotal and experimental evidence of a link between sexual activity, and sedation, increased relaxation and calmness in the post-coital period (Kruger et al., 2002; Brody,

2006), which is likely to be mediated, at least in part, by an activated OXT system. As OXT was shown to exert reinforcing and rewarding actions (Liberzon et al., 1997), the possibility further exists that enforced and reinforced trust to the sexual partner also involves brain OXT (Kosfeld et al., 2005), although this is still highly speculative.

Taken together, the release of the neuropeptide OXT within distinct brain regions during close social interactions, like suckling the offspring in lactating mammals or sexual activity in males, is not only involved in the regulation of this particular social behaviour, but also in beneficial effects of pro-social interactions (Fig. 3). Such positive effects are likely to involve reduced anxiety levels, attenuated stress responses, increased calmness and sedation, as seen both in lactation and after mating (Carter et al., 2001; Neumann, 2002; Heinrichs et al., 2003; Waldherr and Neumann, 2007).

Conclusions

The experimental examples provided here indicate that intracerebral microdialysis and retrodialysis are valuable techniques to gain information on dynamic changes in local AVP and OXT release during the display of social behaviours and its behavioural relevance/importance. Alterations in the local release of these neuropeptides could be demonstrated in a peptide-specific manner in relevant brain regions, including the lateral septum, hypothalamic PVN and central amygdala, during the display of, e.g. intermale and maternal aggression and mating. Manipulations of local AVP or OXT activity revealed two distinct modes of actions of these neuropeptides. Changes in local neuropeptide release during exposure to a social stimulus may be importantly involved in (1) the acute regulation of context-dependent social behaviours and (2) the rather long-term regulation of other related behaviours which are likely to be relevant to the social context, including anxiety, reward or social cognition (Fig. 3). For example, during the maternal defence test, an increase in local OXT release (PVN, amygdala) in lactating dams could be directly correlated with the intensity of maternal aggression. In contrast, during intermale aggression, changes in septal AVP release were not found to be directly linked with the level of aggression, but rather with the acute regulation of non-aggressive social interactions and of anxiety-related behaviours. However, these related behaviours, in turn, may play a role in the fine-tuned regulation of aggression during subsequent interactions with an aggressive conspecific. Moreover, sexual behaviour and mating in males is accompanied by intra-PVN OXT release, which is likely to be directly or indirectly involved in promoting and reinforcing sexual activities as well as in subsequent, rather long-term anti-stress effects, such as anxiolysis.

Still, there is limited knowledge about the functional implications of observed differences in the dynamics of neuropeptide release among mammalian species, sexes or individuals. Furthermore, to understand the regulatory capacities of AVP and OXT within the brain on a neuronal level, it is essential to investigate the neuronal mechanisms and signalling pathways involved in their receptor-mediated behavioural actions (Huber et al., 2005; Allaman-Exertier et al., 2007; Blume et al., 2008). Understanding their function in a social context may provide insights into the wide variations in social organizations among species. Moreover, it may help explain inter-individual variations in the wide spectrum of human social behaviours. Such insights are also likely to be important for the development of appropriate treatments of human social disorders, like social phobia and autism spectrum disorders, but also of violence and other forms of antisocial behaviours.

Abbreviations

AVP	arginine vasopressin
AVT	arginine vasotocin
BNST	bed nucleus of the stria terminalis
HAB	high anxiety-related behaviour
LAB	low anxiety-related behaviour
NAB	normal anxiety-related behaviour
OXT	oxytocin
OXTR	oxytocin receptor

PVN	paraventricular nucleus
SON	supraoptic nucleus
V1aR	AVP V1a receptor
V1bR	AVP V1b receptor

Acknowledgements

We are grateful to Dr. M. Manning and Dr. R. Landgraf for their support throughout the years for generously providing the vasopressin and oxytocin receptor antagonists and for radioimmunological quantification of neuropeptides, respectively.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 23

Interactions between dopamine and oxytocin in the control of sexual behaviour

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Abstract: Dopamine and oxytocin are two key neuromodulators involved in reproductive behaviours, such as mating and maternal care. Much evidence underlies their separate roles in such behaviours, but particularly in sexual behaviour. It is generally believed that central dopaminergic and oxytocinergic systems work together to regulate the expression of penile erection, but relatively little is known regarding how they interact. Thus, this review aims to discuss neuroanatomical proof, neuromodulator secretory profiles in the hypothalamus and behavioural pharmacological evidence which support a dopamine–oxytocin link in three hypothalamic nuclei that have been implicated in sexual behaviour, namely the medial preoptic nucleus, supraoptic nucleus and paraventricular nucleus (PVN). We also aim to provide an overview of potential dopamine-mediated transduction pathways that occur within these nuclei and are correlated with the exhibition of penile erection. The PVN provides the most convincing evidence for a dopamine–oxytocin link and it is becoming increasingly apparent that parvocellular oxytocinergic neurons in the PVN, in part, mediate the effects of dopamine to elicit penile erection. However, while we show that oxytocin neurons express dopamine receptors, other evidence on whether dopaminergic activation of PVN oxytocin cells involves a direct and/or indirect mechanism is inconclusive and further evidence is required to establish whether the two systems interact synergistically or sequentially in the regulation of penile erection.

Keywords: D2-like dopamine receptors; medial preoptic nucleus; oxytocin; paraventricular nucleus; sexual behaviour; supraoptic nucleus

Introduction

Reproductive behaviours comprise the repeated and ever-changing tide of social interaction from sexual arousal and copulation, to birth and maternal care. Many neurotransmitters and neuropeptides have been implicated in the neuroendocrine control of these behaviours and their complex interactions in the brain underlie complex emotional and motor responses (Lopez et al., 1999; Giuliano and Allard for review, 2001; Johns et al., 2005). The neuropeptide, oxytocin (OXT), and the monoamine, dopamine, are among the most studied neuromodulators involved in these behaviours so far (Argiolas and Melis, 2004; Paredes and Agmo, 2004; Bancroft, 2005). It is believed that dopaminergic and oxytocinergic pathways form a complex neural circuitry that may subserve a range of social behaviours that rely on the integration of social and olfactory cues

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DOI: 10.1016/S0079-6123(08)00423-8

with behaviourally specific cues. In this paper we will briefly outline the separate roles of OXT and dopamine before critically reviewing the evidence for whether dopamine controls OXT mechanisms during reproductive behaviour.

Hypothalamic nuclei mediating reproductive behaviours

While brain regions such as the ventral tegmental area (VTA), periaqeductal grey and prefrontal cortex are important in performing reproductive behaviours, it is the hypothalamus which evidently integrates incoming sensory and peripheral signals and co-ordinates the behavioural response. The hypothalamus comprises many nuclei, each of which may be further divided into subregions of phenotypically similar cells. However, evidence at present indicates the involvement of just a few of these regions in reproductive behaviours. The medial preoptic area (MPOA) is one extensively investigated region that is strongly implicated in many reproductive behaviours, including sexual behaviour and maternal care — its lesion in males and females can prevent some key elements of sexual behaviour and in females disrupts maternal behaviour. The adjacent paraventricular nucleus (PVN) also plays an important, if modulatory, facilitatory role — e.g. it mediates penile erection in males, onset of maternal behaviour and responses to social/affiliative stimuli. Other important hypothalamic nuclei in sexual and maternal behaviour include the ventromedial nucleus (VMN, particularly in female sexual behaviour), and evidence is emerging for a role for the supraoptic nucleus (SON) in various behaviours, including sexual interaction (Pattij et al., 2005; Caquineau et al., 2006). Together, in a way that has not yet been fully elucidated, these regions co-ordinate appropriate physiological, social and emotional responses that comprise typical behaviour patterns (Numan and Insel, 2003; Paredes and Agmo, 2004 for overview).

The role of oxytocin

While OXT secreted peripherally has a classic role in parturition and milk ejection (Russell et al., 2003), OXT also mediates sexual, affiliative and maternal behaviours (Argiolas and Melis, 1995, 2004; Insel, 1997; Insel et al., 1997; Argiolas, 1999; Kendrick, 2004; Lonstein and Morrell, 2006). OXT is released and acts in the brain to facilitate these social interactions. The richest sources of OXT mediating behaviours are the terminals of centrally projecting PVN neurons or the dendrites of magnocellular OXT neurons in the PVN and SON (Ludwig, 1998); a comparatively small population of OXT neurons is also located within the median preoptic nucleus (MPN) subregion of the MPOA. These OXT neurons are recruited and release OXT during reproductive behaviours in both female and male animals, including rodents and squirrel monkeys, acting via the oxytocin receptor (OXTR) which is distributed widely in hypothalamic, limbic, brainstem and spinal cord regions. While there is a substantial literature on the role of OXT in a variety of behaviours, here we intend to focus on sexual behaviour.

Oxytocin and sexual behaviour

OXT is implicated in the facilitation of sexual behaviour in both females and males. Although OXT release into the blood increases during sexual arousal and copulation in rodents and humans (review by Argiolas and Melis, 2004; Bancroft, 2005), it is OXT action within the brain that plays an important role in the suite of behaviours that define a sexual encounter, as shown by studies in a variety of species. In males, central OXT is acutely implicated in both penile erection and ejaculation mechanisms (Veronneau-Longueville et al., 1999; Argiolas and Melis, 2004; Coolen et al., 2004; Donatucci, 2006). In females, evidence supports OXT action specifically in proceptive behaviour and lordosis (Pedersen and Boccia, 2006).

We know that OXT neurons in the hypothalamus are recruited since PVN and SON OXT neurons become activated, expressing Fos during various stages of copulation (Witt and Insel, 1994; Pattij et al., 2005), including at presentation of mate cues with further enhancement during intromission and copulation. Indeed, electrical activity of the penile/pelvic nerves increases the firing rate of OXT neurons (Yanagimoto et al., 1996), suggesting that peripheral signals feed into the brain control of these behaviours. Further indications that OXT

neurons are activated include the increased release of OXT in the mediobasal hypothalamus of female sheep during intromission (Kendrick et al., 1993). Likewise in male rats, increased OXT levels within the PVN have been correlated with mating behaviour (Waldherr and Neumann, 2007). However, OXT release within the MPOA and SON has not yet been reported for either males or females during sexual behaviour. Thus, it seems clear that particularly the PVN and perhaps the MPOA and SON are three potentially important sites of action during sexual pairing. OXT administration into these regions induces receptivity in female hamsters and rats and penile erection in male rats and mice that is prevented by pre-lesioning the PVN or intra-nuclear administration of OXTR antagonist (Argiolas, 1999). Also, when paired with a mate, sexual behaviours in males and females are delayed by discrete microinjection of OXT antagonist into the regions (Argiolas and Melis, 2004, 2005; Bancroft, 2005 for review). Further evidence for a key role for OXT includes impaired sexual behaviours in rats with attenuated OXT expression in the PVN and SON, at least in males (see Argiolas, 1999 for review; Pattij et al., 2005). Interestingly, social interaction alone induces OXT release within various brain regions (Landgraf and Neumann, 2004) and there is impaired social and affiliative behaviour in mice lacking OXT or OXTR (Takayanagi et al., 2005), which may indicate a general permissive role for OXT in reproductive behaviour.

The sites of action of OXT during the various sexual behaviours are not yet clear. Evidently OXT has an autoregulatory action on PVN OXT neurons, as indicated above, but OXT neurons also project to various regions of the central nervous system, including the spinal cord centres mediating penile erection (Veronneau-Longueville et al., 1999) and the VMN mediating lordosis (Whitman and Albers, 1998), as well as the MPOA, which is often reported to be responsible for arousal and receptivity in both sexes (Whitman and Albers, 1997). Furthermore, OXT antagonist administration into the cerebral ventricles allowing diffusion and action at distant sites also delays copulatory (intromission and lordosis) behaviours in both males and females (Giraldi et al., 2004; Argiolas and Melis, 2005). Therefore, the wide distribution of OXT fibres, release and action probably underlie a variety of roles that it plays in sexual behaviours, ranging from motivation, through arousal, to consummation.

Mechanisms recruiting OXT neurons during arousal and copulation are poorly understood in both sexes. Some evidence points to inhibitory and excitatory roles for other neuropeptides. Vasopressin, a similar nonapeptide to OXT produced in adjacent neurons, and opioids inhibit OXT mechanisms (Argiolas and Melis, 2004; Pedersen and Boccia, 2006) providing a restraining control, while alpha melanocyte stimulating hormone (αMSH) activates SON OXT neurons and potently induces penile erection and male sexual behaviour in male rats (Caquineau et al., 2006). Additionally, the serotonergic system is believed to modulate OXT release during ejaculation (De Jong et al., 2007). While, information on such feed-forward mechanisms during sexual behaviours is currently limited, many neurotransmitters and neuromodulators are known to regulate OXT neurons under other physiological conditions and behaviours. Major regulatory pathways to OXT neurons include the monoamines, of which dopamine is a potentially important input. Dopamine release and action has been extensively studied in the context of reproductive behaviours, including sexual behaviour, and the next section gives a brief overview of its key roles.

The role of dopamine

Dopamine is an important central neurotransmitter that is involved in many behavioural and integrative functions within the central nervous system. The enzyme tyrosine hydroxylase converts tyrosine to dopamine, which is located in high concentrations in many brain regions such as the striatum, substantia nigra and nucleus accumbens where it is involved in a range of functions including cognition, arousal, co-ordination and motricity. Dopamine acts via five dopamine receptors which fall into two main categories: the D1-like receptors (D1 and D5) and the D2-like receptors (D2, D3 and D4), which are also widely distributed around the

brain and spinal cord. Dopamine and its receptors have been implicated in many behaviours including sexual, affiliative and maternal behaviour (Numan and Insel, 2003; Paredes and Agmo, 2004 for overview). One key source of dopamine mediating behaviours is thought to be the A13/A14/A15 nuclei of the incertohypothalamic dopamine system, which innervate the hypothalamic nuclei involved in social interaction and behavioural responses, although the tuberoinfundibular dopamine neurons may also have a role indirectly via their regulation of prolactin secretion which in some species acts as a gonadotrophin and drives sex steroid secretion. Additionally there are dopaminergic neurons located within the PVN (Jourdain et al., 1999) that are part of a local microcircuit that may be important in neuroendocrine PVN neuron activity. However, as some authors have pointed out, care must be taken to establish whether dopamine actions during behaviours are simply allied to its major roles in motor function and reward or have a precise and identified (separate) role in the three identified key hypothalamic nuclei (the MPOA, PVN and SON), influencing well-described aspects of behavioural control. While there have been concerns that dopamine does not play a specific role in sexual behaviour, convincing evidence that it importantly contributes is now accumulating as outlined below.

Dopamine and sexual behaviour

Recent studies have drawn much attention to the potential physiological role of dopamine in the facilitation of sexual behaviour (Argiolas and Melis, 1995; Giuliano and Allard, 2001; Paredes and Agmo, 2004), including in humans (Kruger et al., 2005). In female rodents dopamine action in the brain is tentatively associated with lordosis, shown by agonist, antagonist and dopamine release studies; but Paredes and Agmo (2004) argue that no specific role can be attributed since dopamine release and action generally have not been assessed in the hypothalamic regions known to mediate female sexual behaviours, such as the MPOA and VMN. However in males the evidence is more concrete (Bancroft, 2005; Donatucci, 2006; Kita et al., 2006). A key finding is the release of dopamine in the hypothalamic regions mediating male sexual behaviour, the MPOA (Sato et al., 1995) and PVN (Melis et al., 2003), with precise timing in relation to exposure to a receptive mate and further enhanced release during copulation (Melis et al., 2003). Dopamine agonists clearly elicit penile erection and accelerate sexual interaction when paired with a mate, effects which are blocked by central dopamine antagonist administration (Brioni et al., 2004; Hsieh et al., 2004; Paredes and Agmo, 2004) and these treatments are most effective when given directly into the PVN or MPOA. The above studies do not reveal whether endogenous dopamine is involved or at which subtype they might be acting. However, one study has reported that dopamine D1 antagonist given systemically decreased intromission frequency in paired sexually naïve males concomitantly with attenuated copulation-induced Fos expression in MPOA neurons (Lumley and Hull, 1999), suggesting a role for endogenous dopamine in the MPOA at intromission. However, the same dose also impairs sucrose intake, which may indicate impaired reward systems (Panocka et al., 1995). Therefore, a specific role for the D1 receptor in sexual behaviour is not yet clear; and most literature indicates a more likely role for the D2-like receptors in MPOA and/or PVN, rather than D1 receptors in mediating these behaviours.

The target neurons in the hypothalamus for dopamine facilitation of precise components of sexual behaviour (i.e. other than motor function or reward) remain elusive. Although many brain areas that receive dopaminergic innervation have been implicated in various components of sexual behaviour, and dopamine receptor expression has been identified in some of these areas, the phenotype of receptive cells in these regions are unknown. So, in the above identified key targets for dopamine action during sexual behaviour, such as the PVN and MPOA, precise dopamine receptor expression patterns are not yet reported. Dopamine also influences SON neurons, although dopamine action in the SON has not been reported during sexual behaviour. So evidence suggests that dopamine acts in the MPOA, PVN and SON, which are also the principal sites of OXT synthesis, release and action. Thus, these three hypothalamic nuclei

make interesting candidates for potential integration sites of central dopamine and OXT neurotransmission in the regulation of male sexual behaviour. If we accept that dopamine release and action plays a specific role in the hypothalamus to facilitate sexual behaviour, at least in male rodents, we can hypothesize that dopamine and OXT exert similar positive effects on male sexual behaviours by non-independent mechanisms. Therefore, we will critically analyse the evidence that dopamine interacts with OXT to mediate its effects. Currently, the PVN shows the most convincing evidence for an interaction between dopamine and OXT in the facilitation of penile erection. However, to our knowledge such a link has not yet been investigated in the MPOA and SON, thus, this review will focus mainly on data obtained from studies on the PVN.

Dopamine–oxytocin link

A variety of technical approaches have been employed to investigate the interaction between central dopamine and OXT neurotransmission during penile erection in the male rat. The evidence falls into three primary categories: neuroanatomical, OXT release and behavioural pharmacological studies; some of which are more convincing than others for a direct dopamine–OXT link. These will be reviewed in the following sections.

Neuroanatomical evidence

At the cellular level, morphological studies have revealed that terminals of dopaminergic neurons originating in the incertohypothalamic system lie in close apposition to somatic and dendritic processes of the magnocellular and parvocellular OXT neurons in the PVN and SON (Buijs et al., 1984; Decavel et al., 1987) which is suggestive of a direct dopaminergic influence. Immunocytochemical studies have revealed abundant expression of D1 (Czyrak et al., 2000) and D4 receptor expression (Defagot et al., 1997; Bitner et al., 2006) in the PVN and SON (Defagot et al., 1997) of male rats; and additionally, D1 and D5 receptors are expressed in the PVN and SON of primate brains (Rivkees and Lachowicz, 1997). However, the neuronal phenotype expressing dopamine receptors remains largely unknown. It is often assumed that dopamine acts on dopamine receptors expressed in OXT cell bodies to elicit penile erection; however, up to now there has been no immunocytochemical confirmation of such a cellular arrangement. For the first time we now show that D2, D3 and D4 receptors separately co-localize with OXT in the MPN, SON and PVN of male rats (Fig. 1), thus revealing the phenotype of at least one set of target neurons in the rat hypothalamus. This supports the hypothesis that endogenous dopamine may bind to and activate OXT cells via D2-like receptors, providing a strong anatomical basis for potentially direct actions of dopamine on OXT cell bodies and dendrites. However, in vivo it has been harder to prove that endogenous dopamine acts on OXT cells.

Oxytocin release studies

One indicator of OXT neuron activity is OXT release, and several reports have demonstrated that dopamine plays a dynamic role in regulating this. Early in vitro studies conducted in the 1970s demonstrated that dopamine and the dopamine receptor antagonist, haloperidol, were able to significantly increase and decrease OXT release, respectively, in hypothalamic explants. Moreover, intracerebroventricular (i.c.v.) or peripheral administration of dopamine or the non-selective dopaminergic agonist apomorphine increased release of OXT into the blood, the hypothalamus and at other extrahypothalamic sites in various rat models and in monkeys (Bridges et al., 1976; Melis et al., 1990, 1992; Cameron et al., 1992; Argiolas, 1999). It is unclear as to which dopamine receptor(s) are involved in central OXT release. Electrophysiological studies may shed some light on this: the D2/D3 receptor agonist quinpirole, but not the D1 agonist SKF38393, increased the excitability of OXT neurons in the SON. In the same study dopamine-induced depolarization was antagonized by the D2-like preferring receptor antagonists, sulpiride and spiperone (Yang et al., 1991), implicating the involvement of D2-like receptors in the excitation of supraoptic OXT neurons. However,

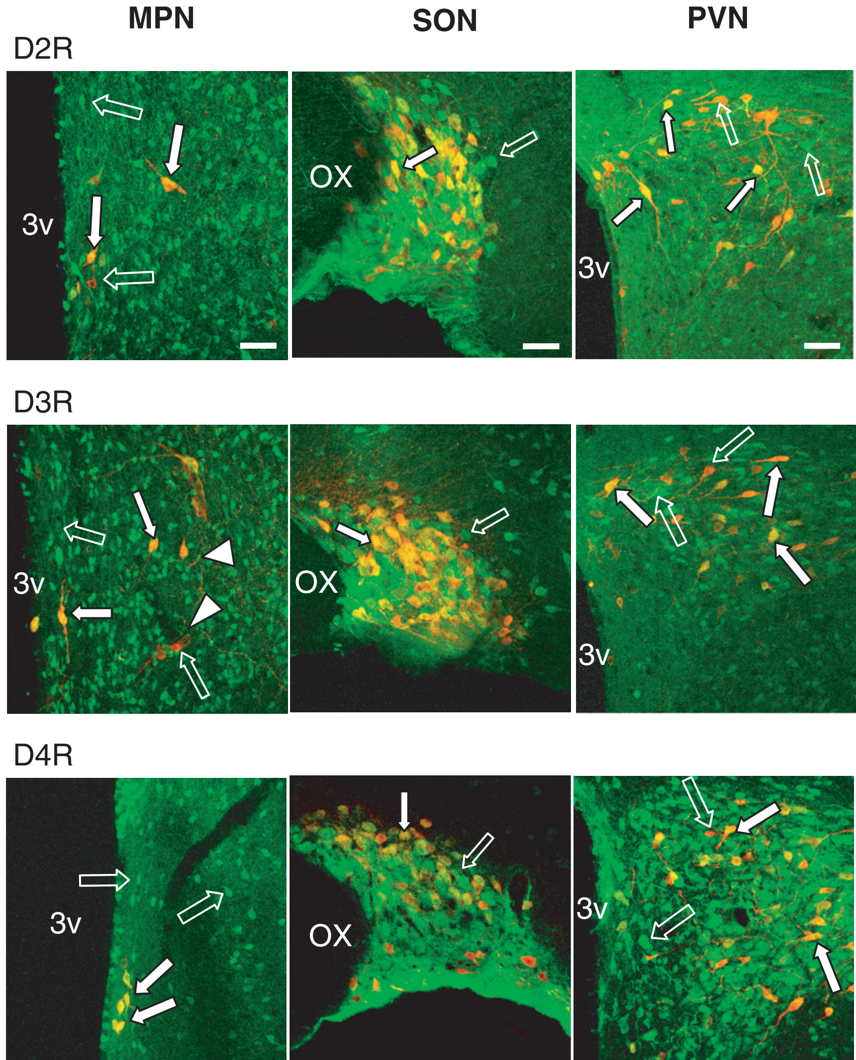

Fig. 1. Expression of dopamine D2, D3 and D4 receptors and oxytocin (OXT) in the MPN, SON and PVN of untreated sexually experienced male rats (dopamine receptor staining is shown in green and OXT peptide staining is shown in red). The D2, D3 and D4 receptors were shown separately to co-localize with OXT in the MPN, SON and PVN. All D2-like receptors were distributed in the cell membrane and the cytosol of OXT neurons. Additionally, D3 receptors were found to be expressed on the dendritic processes of MPN OXT neurons. Filled arrows indicate co-expression of dopamine receptor and OXT, empty arrows show dopamine receptor or OXT staining only and arrowheads illustrate dopamine receptor and OXT co-localization on dendritic processes (Scale bars represent 100 μm and are the same for D3 and D4 receptor and OXT images as are for the D2 receptor and OXT.). 3v = third ventricle; OX = optic chiasm. (See Color Plate 23.1 in color plate section.)

firing rate changes are not necessarily correlated with dendritic OXT release (Ludwig, 1998). As previously stated, OXT neurons in the hypothalamus are innervated by incertohypothalamic-derived dopaminergic fibres, although magnocellular OXT cells (in the PVN) are also innervated by intra-PVN dopaminergic fibres (Jourdain et al., 1999). Thus, there could be a predominantly intra-PVN

dopaminergic innervation of magnocellular OXT neurons and, conversely, an incertohypothalamic innervation of parvocellular OXT neurons, which may help to explain the apparent differential involvement of these neurons in sexual behaviour, where magnocellular OXT neurons are implicated in intromission and ejaculation (Pattij et al., 2005; Caquineau et al., 2006) and parvocellular OXT neurons are implicated in penile erection (Witt and Insel, 1994; Kita et al., 2006).

So, the above evidence indicates that D1, D2, D3 and D4 receptors are present in the MPN, SON and PVN in rats. Although the neuronal phenotype expressing these receptors is unclear, at least some are oxytocinergic. Such an anatomical arrangement provides support for a potential dopamine–OXT link in hypothalamic nuclei. It could be postulated that endogenous dopamine may act via D1-like and/or D2-like receptors in the MPN, SON and PVN to activate the hypothalamic OXT system. Potentially important sites for the subsequent release of OXT include the PVN, hippocampus (Melis et al., 1992) and spinal cord (Giuliano et al., 2001).

Behavioural pharmacological evidence

The proposed inter-dependent relationship between dopamine and OXT neurons in the PVN during penile erection was substantiated by the attenuation of apomorphine-induced penile erection after bilateral lesioning of the PVN (Argiolas et al., 1987a) that depletes central OXT levels in extra-hypothalamic brain areas (Hawthorn et al., 1985). Stimulation of penile erection by apomorphine or the selective D4 receptor agonist, PD 168077 is also prevented by i.c.v., but not intra-PVN, OXTR antagonist administration (Melis et al., 1992, 2005), suggesting that OXTRs located outwith the PVN are involved in mediating apomorphine-induced penile erection.

Thus, it is not clear as to whether during penile erection, dopamine increases OXT levels in the PVN (via magnocellular cells) and/or at sites outwith the PVN (via parvocellular cells) such as in the spinal cord where pro-erectile centres exist (Veronneau-Longueville et al., 1999) or the hippocampus (Melis et al., 1992). There is increasing evidence to suggest that parvocellular OXT neurons are part of the neural network controlling penile erection (Witt and Insel, 1994; Veronneau-Longueville et al., 1999; Kita et al., 2006). It is generally believed that apomorphine-induced penile erection involves, at least in part, release of OXT at extra-hypothalamic areas via these parvocellular fibres; however, intra-hypothalamic OXT release and action cannot be ruled out, although perhaps intra-PVN oxytocinergic mechanisms could be.

As stated above, dopamine agonist-induced penile erection can be inhibited by an OXTR antagonist (Argiolas et al., 1987b), however, OXT-induced penile erection is not inhibited by the dopamine receptor antagonist, haloperidol (Melis et al., 1997). Thus, dopamine may lie upstream to OXT in the regulation of penile erection but not vice versa. Interestingly, in another study, penile erection elicited by OXT was antagonized by the generally non-selective dopamine receptor antagonist, clozapine (although clozapine does have slightly more affinity for D2-like than D1-like receptors) (Martino et al., 2005). A possible explanation for such a discrepancy in these studies is due to the different pharmacological agents and doses used in the studies (D2-preferring haloperidol (350 nmol) versus D4-preferring clozapine (1–10 μmol)). The blockade of OXT-induced penile erection by clozapine as seen by Martino and colleagues may be due to antagonism of all dopamine receptors and perhaps unknown receptor sites involved in central and peripheral control of erection due to the higher dose used. Therefore there may be a synergistic or dual activation of both the dopaminergic and oxytocinergic systems that may have a parallel involvement in the mediation of penile erection. It is interesting, however, to postulate that D4 receptors may be located downstream and D2 receptors located upstream from OXT cells and that D2 and D4 receptors may differentially regulate penile erection via oxytocinergic and/or non-oxytocinergic pathways.

Many of the current studies support a dopamine–OXT relationship in the PVN in relation to erectile function. However, there are some new findings that implicate other brain nuclei and suggest a complex communication network exists between the PVN and other brain nuclei known to

be involved in sexual behaviour. Apomorphine and PD168077 (D4 receptor agonist) given intra-PVN have been shown to increase the levels of dopamine and its metabolite DOPAC in the nucleus accumbens (NAcc), which occurs concomitantly with penile erection, and both the increase in dopamine and penile erection were attenuated by i.c.v. injection of an OXT antagonist (Succu et al., 2007). The NAcc is generally associated with sexual motivation and sexual arousal, however, a dopamine–OXT–dopamine link may exist between the PVN and NAcc in the regulation of erection. Interestingly, it has recently been suggested that dopamine and OXT neurotransmission form potential underlying neural pathways between the PVN, NAcc and the VTA during penile erection. Paraventricular OXT fibres project to the VTA (Roeling et al., 1993) where OXTRs are also located (Freundmercier et al., 1987). Intra-VTA administration of OXT increased dopamine concentrations in the NAcc and PVN, such increases occurred concomitantly with penile erection. Both of these effects were attenuated after either intra-VTA injection of an OXTR antagonist or intra-NAcc or intra-PVN injection of the dopamine receptor antagonist, haloperidol (Melis et al., 2007). Thus, it seems that OXT in the VTA may stimulate as yet unknown excitatory pathways that project (perhaps indirectly) back to the PVN to enhance dopaminergic neurotransmission and so facilitate penile erection. It can therefore be seen that dopamine-induced pro-erectile effects are not mediated solely by OXT pathways originating in the PVN but by other OXT-containing nuclei that form a much larger and highly integrated network subserving central control of penile erection.

Signalling pathways

It is generally believed that dopamine receptor stimulation in the PVN triggers a cascade of events that involves the activation of parvocellular OXT neurons and the resultant release of OXT at extra-hypothalamic sites. However, the intracellular signalling pathways and transduction mechanisms activated upon dopamine receptor stimulation and how they facilitate OXT release remain to be elucidated. Many studies have shown a wide range of signalling pathways activated by central dopamine or a dopamine receptor agonist (all of which cannot be discussed in this review). It is evident however, that many of the signalling pathways are very much dependent on the brain nucleus they occur in, cell type involved and cell location as well as the G-protein to which the receptor is coupled to. Therefore it is very difficult to ascribe one particular signalling pathway activated by dopamine in one brain area to another. Evidence for direct and indirect dopamine-mediated signalling pathways and those correlated with penile erection in the MPN, SON and PVN will now be discussed.

Potential intracellular signalling in oxytocin cells

Typically, dopamine receptors are comprised of seven transmembrane domain G-protein coupled receptors which belong to one of two families; the D1-like receptors which couple positively to adenylate cyclase and the D2-like receptors which either couple negatively to adenylate cyclase or via other signalling pathways. If the general assumption is that D2-like receptors are involved in OXT-mediated penile erection then it is logical to assume such receptor stimulation would in fact inhibit OXT release. However, it has been suggested that in the PVN, dopamine-induced oxytocinergic activation may involve a calcium dependent nitric oxide (NO) pathway rather than the classical cAMP pathway. Intra-PVN injection of ω-conotoxin-GVIA, a selective antagonist of N-type calcium channels inhibits apomorphine- and OXT-induced penile erection, moreover, blockade of the N-type calcium channels attenuates the increase in nitrite and nitrate concentrations (indicators of NO activity) during penile erection (Succu et al., 1998). The NO precursor, neuronal nitric oxide synthase (NOS) is abundantly expressed in oxytocinergic neurons (Ferrini et al., 2001; Xiao et al., 2005). Centrally administered NOS inhibitors were shown to prevent dopamine agonist and OXT-induced penile erection. Furthermore, the concentration of NO in PVN dialysates increases after administration of apomorphine and D2-like agonists, an effect which occurs concomitantly with penile erection (see Argiolas and Melis, 2005 for review). It has

been suggested that dopamine receptor stimulation initiates the opening of the N-type calcium channels in a similar manner to NMDA receptors i.e. via coupling to a G protein, however, activation of the N-type calcium channels may be via an as yet unknown intracellular signalling pathway. In line with the former hypothesis, such increased intracellular calcium levels would activate NOS which in turn stimulates NO production and the subsequent activation of OXT neurons. In another study, it has been shown that D2-like receptors coupled to the chimeric G-protein, $G_{\alpha q}$ in transfected cell lines increased intracellular calcium levels upon dopamine stimulation (Moreland et al., 2004), although it is not known if the D2-like receptors would naturally couple to this G-protein physiologically. In the neurohypophysis, activation of dopamine D4 receptors leads to inhibition of potassium currents (Wilke et al., 1998) thus D2-like receptors may have a partial inhibitory effect on cell hyperpolarization thereby enhancing OXT release. The D4 receptor agonist, PD-168077 has been shown to induce Fos expression and ERK 1/2 phosphorylation in the PVN of male rats (Bitner et al., 2006). Such an effect was antagonized by A-381393, a dopamine D4 receptor antagonist, and SL327, an ERK 1/2 phosphorylating inhibitor. Additionally, D4 receptor expression was co-localized with Fos in PVN neurons although it is not known if these were oxytocinergic. Unfortunately in this study the gene expression was not correlated with penile erection; however, it does suggest that D4 receptors in the PVN mediate the actions of PD-168077 by activating immediate early gene and/or MAP kinase pathways (Bitner et al., 2006; potential dopaminergic actions on OXT neurons in the PVN are summarized in Fig. 2).

In the MPN there does not appear to be any literature investigating a dopamine–OXT link in relation to sexual behaviour, thus even less is known regarding the potential signalling pathways underlying dopamine–OXT interactions in this nucleus. However, it is believed that NO facilitates dopamine release in the MPOA as shown by a comprehensive study carried out by Sato and Hull (2006) where they postulated that activation of a

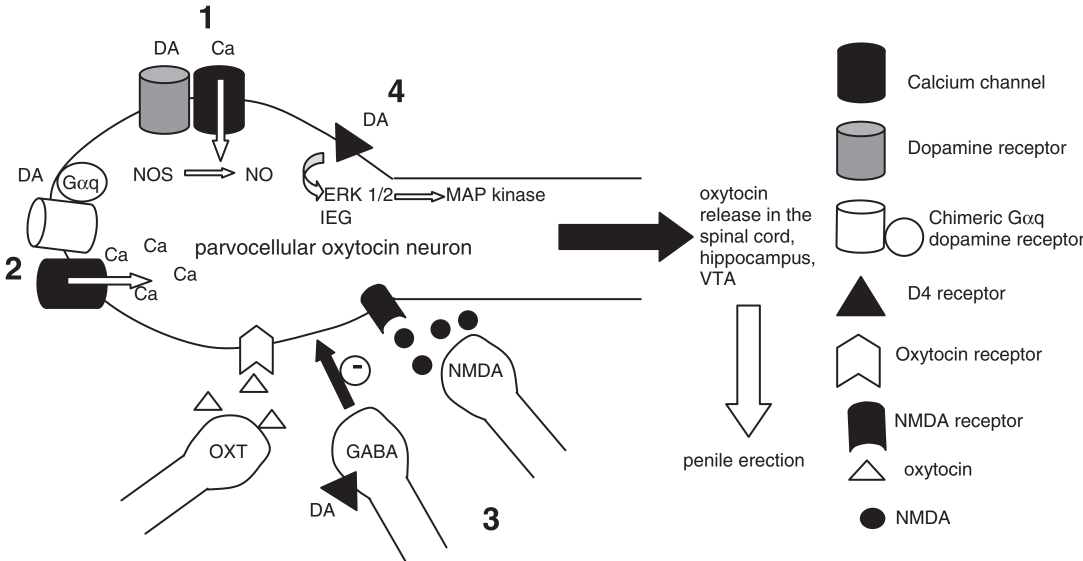

Fig. 2. Summary diagram of dopamine-mediated signalling pathways during penile erection. Dopamine may act via (1) a calcium dependent nitric oxide pathway, (2) the chimeric G-protein Gαq to increase intracellular calcium levels, (3) presynaptic inhibition of GABA neurons via D4 receptors, thus disinhibiting OXT neurons and leaving them free to respond to excitatory neuromediators and (4) D4 receptors to stimulate ERK 1/2 phosphorylation and/or IEG expression to activate MAP kinase pathways, to excite parvocellular oxytocinergic cells and stimulate release of oxytocin in areas such as the spinal cord, hippocampus and VTA.

NO-cGMP pathway enhanced MPOA dopamine release. An analogue of cGMP and an inhibitor of its precursor, guanylate cyclase increased and decreased MPOA dopamine levels, respectively. In addition, cGMP was shown to concomitantly increase dopamine levels and ejaculation frequency; conversely, inhibition of guanylate cyclase decreased ejaculation frequency (Sato and Hull, 2006). This provides strong evidence for a NO–dopamine link in the MPOA during male sexual behaviour; however, as before the neuronal phenotype expressing NO is not known. Because NO can act as an intra- and intercellular messenger and is present in hypothalamic OXT neurons, it could be tempting to suggest an OXT–NO–dopamine pathway in the MPOA.

Signalling via interneurons

It is known that a complex cellular hierarchy exists in the SON and PVN whereby OXT neurons are heavily modulated by inhibitory GABAergic and excitatory glutamatergic neurons which themselves can be influenced by a range of synaptic inputs and neurotransmitters present in the extracellular fluid. GABAergic and glutamatergic neurons synapse with magnocellular OXT soma and dendrites in the PVN and SON whereas dopaminergic innervation of these nuclei is comparatively scarce (Jourdain et al., 1999), thus it seems reasonable to assume that endogenous dopamine could exert its' effect on OXT release by acting indirectly via glutamatergic and/or GABAergic neurons rather than OXT neurons directly. Evidence for an intermediary role for GABA includes that intra-PVN injection of muscimol, the $GABA_A$ receptor agonist, inhibits apomorphine- and OXT-induced penile erections and, in parallel, attenuates the increase in nitrite and nitrate concentrations in the PVN (Melis and Argiolas, 2002). In particular, the D4 receptor has been implicated in the disinhibition of GABAergic and inhibition of glutamatergic neurotransmission in the SON by acting presynaptically (Price and Pittman, 2001; Azdad et al., 2003). Although it is unclear as to which system would predominate under physiological conditions, it could be speculated that dopamine-induced inhibition of GABAergic and glutamatergic neurons would somehow increase the responsiveness of the postsynaptic cell, conferring on it a greater sensitivity, free to respond to other excitatory factors such as OXT. Thus, it could be that during penile erection, dopamine acts presynaptically to disinhibit OXT neurons and so facilitate the activation of the hypothalamic oxytocinergic system. One study which contradicts this GABA theory showed that Zolpidem, a selective receptor agonist for the $GABA_A$ receptor α subunit, significantly induced Fos expression in magnocellular OXT neurons in the PVN; however, this was not correlated with penile erection (Kiss et al., 2006). As previously described, activation of $GABA_A$ receptors on OXT neurons is normally associated with increased GABAergic inhibition and, thus, reduced activity in OXT neurons; but in this study they found increased magnocellular oxytocinergic activation in the PVN but not the SON. Zolpidem also increased Fos expression in the parvocellular PVN but again the neuronal phenotype expressing the Fos was unknown (Kiss et al., 2006). GABA may differentially modulate OXT neurons depending on the nucleus they are expressed in. It is possible that in this study, Zolpidem was activating presynaptic as well as postsynaptic $GABA_A$ receptors which would partly explain the increased Fos expression in OXT neurons. Additionally, $GABA_A$ receptors are comprised of a variety of subunits, each with their own isoforms, thus giving rise to a diverse range of functional $GABA_A$ receptors. It has yet to be demonstrated, but $GABA_A$ receptors activated by Zolpidem in this study may perform in a contradictory manner to conventional $GABA_A$ receptors thereby inhibiting GABAergic neurotransmission.

Thus, there are a range of potential direct and indirect signalling pathways activated upon dopamine receptor stimulation in the MPN, SON and PVN. However, further studies are required to elaborate on the activation of behaviourally specific transduction mechanisms and their correlation with the expression of penile erection.

Conclusion

The central neural networks and intracellular signalling pathways controlling penile erection are

far from being fully established. Evidence shows that dopamine acts at least partly via the oxytocinergic system and the MPN, SON and PVN are three brain regions that are likely sites for dopaminergic control of OXT neurons. However, although OXT neurons express dopamine receptors it is still to be established whether endogenous dopamine acts directly via these to mediate sexual (or other) behaviours: other extrahypothalamic areas mediated by these and other neuromodulators are almost certainly involved. Cross-talk between central dopaminergic and oxytocinergic neurotransmission in the mediation of penile erection appears to be an exciting and intriguing prospect which warrants further investigation. Due to the high incidence of stereotypical side effects associated with erectile dysfunction drugs that modulate dopaminergic transmission, it would be interesting to further investigate how central dopamine and OXT pathways interact. Such dual participation of dopamine and OXT in the control of penile erection could be clinically exploited; particularly the oxytocinergic system, which is a key therapeutic target due to its pro-erectile effects and seemingly lack of ability to cause adverse side effects.

Abbreviations

D1, D2, D3, D4, D5	dopamine receptor subtypes 1–5
i.c.v.	intracerebroventricular
MPOA	medial preoptic area
MPN	median preoptic nucleus
NAcc	nucleus accumbens
NO	nitric oxide
NOS	nitric oxide synthase
OXT	oxytocin
OXTR	oxytocin receptor
PVN	paraventricular nucleus
SON	supraoptic nucleus
VMN	ventromedial nucleus
VTA	ventral tegmental area

Acknowledgements

We thank Mel Addison and Vicky Tobin for their valuable help in performing immunofluorescence and Pfizer Pharmaceuticals and the BBSRC for a CASE Studentship award to Tracey A. Baskerville.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 24

Steroidal/neuropeptide interactions in hypothalamus and amygdala related to social anxiety

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Abstract: Oestrogens (E) influence the activity of oxytocin (OT) producing neurons and heighten the rate of transcription of the oxytocin receptor (OTR) gene. Working through synergistic activities of two different oestrogen receptors (ERs), likely gene duplication products, in the hypothalamus and amygdala, E supports social recognition by mice. As part of social recognition and approach, it is important for mice to assess the risks of social interactions, thus to reduce the social anxiety. Here we argue that hyperactivity in ascending central nervous system arousal systems would work in the opposite direction: increasing social anxiety by potentiating fear-related mechanisms in the amygdala. In humans, such increased social anxiety might account for some features of autism.

Keywords: oestrogen; estradiol; neuropeptide; oxytocin; hypothalamus; paraventricular; amygdala; anxiety; autism

Introduction

The involvement of gonadal steroid hormones in the regulation of both social behaviour and anxiety makes them good candidates for the investigation of the interplay between gonadal hormones and those human disorders that are characterized by abnormal social behaviour. This includes autism spectrum disorder, schizophrenia and schizotypical disorders, social phobias, social anxiety as well as hyperaggression and violence. Most, if not all, of these disorders manifest themselves in a highly sexually dimorphic manner. In the case of autism and schizophrenia, the incidence in males is significantly higher than in females. Not only do males suffer more from these disorders, also, they suffer from more severe forms of the disease (Nicole et al., 1992; Knickmeyer and Baron-Cohen, 2006). Hyperaggression and violence are widely known to be significantly more expressed in males than in females. Females, thus, appear somewhat protected from disorders of sociality, suggesting a pro-social effect of the female gonadal hormones, oestrogens and progesterone.

Gonadal hormones act in the body primarily through ligand-regulated nuclear transcription factors, regulating and/or modulating the activity of hundreds of genes both in the periphery and in the central nervous system (CNS). Among these genes are those related to the production or action

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DOI: 10.1016/S0079-6123(08)00424-X

of a number of neurotransmitters that have been implicated in social behaviour (see below).

Here we will focus on the interactions of key neuropeptides, namely oxytocin (OT), vasopressin (VP) and opioids, with oestrogens and their combined involvement in the regulation and/or modulation of social anxiety through amydalar actions. We then speculate on how understanding this regulation of social anxiety can aid in the resolution of disorders affecting predominantly the social domain and provide insights into the mechanisms underlying their sexually dimorphic incidence.

ERα compared to ERβ

The main modes of action of oestrogens are through two nuclear receptors, oestrogen receptor alpha (ERα) and oestrogen receptor beta (ERβ). Likely the product of gene duplication (Kuiper et al., 1996), the two ERs are encoded by different genes and are both involved in the regulation of physiology and behaviour (Heldring et al., 2007). Investigations with mice lacking either ERα, or ERβ, or both (α-ERKO, β-ERKO and αβ-ERKO) have demonstrated that the two receptor subtypes often play different roles in the action of oestrogens and several co-activators. The often non-overlapping distribution of the receptors (Shugrue et al., 1997), together with direct inhibitory action of ERβ on the gene transcription regulatory activity of ERα, may explain why on several occasions the action of the two ERs has been described as opposite (reviewed in Matthews and Gustafsson, 2003).

As it turns out, most social behaviours, from mating to aggression, social recognition and social learning are differently regulated by the two ERs, with their involvement being in opposite directions in most cases.

Male α-ERKO mice are infertile and do not show normal sexual behaviour (Ogawa et al., 1998, 2000; Couse and Korach, 1999), while female α-ERKO mice show masculinized behavioural responses towards males and fail to show proceptive or receptive mating responses towards a stud male. In fact, they are treated like a male by other mice, possibly in response to their atypical female behaviour (Ogawa et al., 1996). Furthermore, in response to a pup placed in their cage α-ERKO females show markedly reduced maternal pup-retrieving and elevated levels of infanticide (Ogawa et al., 1998). Conversely, β-ERKO male and female mice do successfully mate and reproduce (Ogawa et al., 1999). Overall, thus, ERα but not ERβ, is necessary for the normal expression of natural reproductive behaviours in both sexes.

Aggressive behaviour too, is differently regulated by ERα and ERβ (reviewed in Ogawa et al., 2004). Male α-ERKO mice show no territorial offensive aggression towards a male intruder to their cage (Ogawa et al., 1998). Females α-ERKO mice, instead, show heightened aggression (Ogawa et al., 1998). The aggressive behavioural profile of the β-ERKO mice is opposite to that of the α-ERKOs. Male β-ERKOs show increased aggression, while female β-ERKOs show reduced testosterone-facilitated offensive aggression but increased maternal defensive aggression (Nomura et al., 2002). These studies show that not only the two ERs play opposite roles in the regulation of aggression, but also their involvement is different in male and female offensive and defensive aggression.

α-ERKO and β-ERKO mice have been studied in a prominent aspect of social behaviour, social recognition, the ability to recognize individual conspecifics, which is crucial for the normal expression of several social behaviours (Choleris et al., 2004). Initial studies suggested that both ERs play a facilitatory role, with both α-ERKO and β-ERKO mice being impaired in this behaviour. Compared to control animals, the KO mice are unable to recognize that a new intruder mouse has been introduced in their home cage, rather than a mouse with which they should have become familiar (Imwalle et al., 2002; Choleris et al., 2003). However, results of a subsequent study using a more sensitive behavioural paradigm, where the mice are given a direct choice between a novel and a familiar conspecific, suggest that the two receptors may actually play different roles. While the α-ERKO mice are completely impaired, the β-ERKO mice can discriminate between the two conspecifics (i.e. they investigate more the novel

than the familiar mouse), even though they do this less well than wild-type mice (Choleris et al., 2006). Thus, ERα appears to be necessary for social recognition, while ERβ only facilitates it. Another type of evidence regarding social behaviour also bears on the issue. Recent studies have shown that pharmacological activation of ERα blocks, while activation of ERβ prolongs, a socially learned food preference. Ovariectomized mice treated with an ERα agonist fail to show a preference for the food their cage mate has just consumed, while mice that received an ERβ agonist not only show a normal socially acquired food preference but also they display that preference for at least twice as long as control mice (Clipperton et al., 2007). This suggests that the opposite action of the two ERs proposed for spatial learning (Rhodes and Frye, 2006) extends also to an exquisitely social form of learning.

This conclusion that ERα and ERβ participate differently in the regulation of social behaviours was reinforced by recent findings showing a differential impairment of female-induced boldness and risk taking by α-ERKO and β-ERKO male mice (Kavaliers et al., 2007). In several species, including that of humans, male risk taking and boldness are increased by female-related cues (Kavaliers et al., 2001). In the presence of a novel sexually accessible female or associated cues, males become "emboldened" making riskier decisions. In mice, this emboldening can be expressed as a reduced response to predator threat. ERα and ERβ mediate different aspects of this emboldening. α-ERKO male mice fail to become emboldened, while β-ERKO males display a heightened level of emboldening and do not distinguish between novel and familiar females. This suggests that ERα and ERβ male have different roles in the determination of "personality" and boldness with ERα necessary for both social responsiveness and recognition and ERβ having a facilitatory role on social recognition.

The impairment of the α-ERKO and β-ERKO mice in social recognition is also reflected in their lack of capability to recognize, avoid and develop aversive responses to parasitized conspecifics (Kavaliers et al., 2003). As a major cost of social behaviour is the increased risk of exposure to parasites and pathogens, the ability to recognize infected individuals is critical. Both male and female mice discriminate, avoid and display aversive responses to infected individuals and cues associated with them. These aversive responses are further modulated by prior familiarity, with mice displaying dampened immuno-compromising aversive and stress responses to familiar infected individuals. Despite this effect of familiarity on stress responses, mice still show similar avoidance of infected individuals, whether familiar or unfamiliar. α-ERKO and β-ERKO mice, however, display reduced avoidance and aversive responses to infected conspecifics and fail to distinguish between familiar and unfamiliar infected individuals. These findings further reinforce the involvement of ERα and ERβ in the modulation of social recognition and social responsiveness.

Social recognition and autism

In humans the proper processing and recognition of facial cues is crucial to the expression of normal social behaviour. One of the hallmarks of autism and related disorders is an impairment of processing and recognition of facial expressions. Patients with Asperger's disorder and the socio-emotional disorder are at increased risk of prosopagnosia, that is the failure to recognize familiar faces (Barton et al., 2004). Furthermore, when faced with a face-recognition task, adults with autism spectrum disorder utilize different face-scanning strategies (i.e. they look at the eyes and other inner features of faces less than normal individuals) and fail to show proper activation of the fusiform face area and regions of the social brain, including the mirror neuron system and the amygdala. The degree of hypoactivation of these areas correlates with social symptoms of autism (Hadjikhani et al., 2007). When they do look at the eyes during facial discriminations task autistic individuals show greater activation of the amygdala, suggesting increased emotional responses associated with gaze fixation (Dalton et al., 2005). Furthermore, when asked to identify the expression of feelings in photos of eyes, autistic patients' lower performance was associated with lower or no

activation of the superior temporal gyrus and the amygdala (Davidson and Slagter, 2000). It seems, thus, that autistic individuals' impaired social recognition is associated with impaired proper processing of social-emotions by the amygdala.

Consistent with the non-human animal literature (Choleris et al., 2006) it appears that in humans too, social recognition is under the control of oestrogens. In this regard, there is a female advantage in facial processing and the recognition of emotional expressions (Montagne et al., 2005; Hampson et al., 2006) that is modulated by testosterone (van Honk and Schutter, 2007). Augmented testosterone (and possibly reduced estradiol) reduces facial recognition and processing. This sex difference in facial recognition and processing has interesting parallels and implications for ERs and sex differences in autism.

E/OT interactions

A neuropeptide that has been involved in all of the oestrogen-dependent social behaviours described above is the nonapeptide OT. OT is produced in the hypothalamus and released in various areas of the brain as well as in the blood stream, thus exerting its effects both in the CNS and in the periphery (Gimpl and Fahrenholz, 2001). Its release from dendrites as well as axons has been studied in some detail (reviewed in Landgraf and Neumann, 2004). In particular, OT is known to foster pro-social behaviours including social recognition (Fergusson, 2000, 2001; Choleris et al., 2003, 2006, 2007), social learning (Popik and van Ree, 1993, 1998), maternal (Bales and Carter, 2003; Pedersen et al., 1994; Insel and Hulihan, 1995; Young et al., 1997; Cho et al., 1999; Razzoli et al., 2003) and sexual behaviour (Carter, 1992; Bancroft, 2005). OT is involved in social bonds — romantic and maternal love — even in humans (Loup et al., 1991; Bartel and Zeki, 2004). Aggression, in contrast, is inhibited by OT administration (McCarthy, 1990; Ferris, 2005) and increased by blocking OT action (Giovenardi et al., 1998; Lubin et al., 2003). OTKO mice are more aggressive in both home cage (Winslow et al., 2000) and semi-natural environment testing condition (Ragnauth et al., 2005).

Consistent with their similar behavioural profiles, OT and oestrogens act in a tightly interrelated manner, with OT activity being regulated by oestrogens at two levels. First, *production of OT* is under the control of oestrogens as indicated by several pieces of evidence. Plasma OT levels and oxytocin receptor (OTR) mRNA fluctuate with the oestrous cycle in a manner consistent with fluctuations in circulating levels of oestrogens (Ho and Lee, 1992; Sarkar et al., 1992; Bale et al., 1995). More direct evidence shows that oestrogens administration heightens the electrical excitability of OT-producing neurons in the paraventricular nucleus (PVN) of the hypothalamus (Akaishi and Sakuma, 1985). Second, the transcription of the gene for the *OTR* is under oestrogen control, with oestrogens administration increasing the rate of transcription from the OTR gene (Quiñones-Jenab et al., 1997). This effect is pronounced in the amygdala, which is relevant for the focus of this review, as highlighted below.

A functional genomic network supporting social recognition

The evidence that the risk of developing an autism spectrum disorder carries important genetic influences is overwhelming (Freitag, 2007; Hoekstra et al., 2007; Losh and Piven, 2007; Szatmari et al., 2007). Our concern is to use our and others' functional genomic evidence to look into the identification of specific genes contributing to autism's component functions: social recognition and the related function, social anxiety.

In this story, OT and its receptor will play major parts, while VP and its receptors will also provide interesting points. The release of OT in the CNS — not only from synaptic endings but also from dendrites — in the hypothalamus and in the amygdala is thought to be of major importance for a variety of biologically adaptive social behaviours (Landgraf and Neumann, 2004).

OT produced within neurons of PVN can be transported along axons into the amygdala (Sofroniew, 1983) where significant levels of OTR

are to be found (Elands et al., 1988; Yoshimura et al., 1993). There, both OT and VP affect neuronal excitability, the two neuropeptides acting on distinct populations of cells (Huber et al., 2005; Terenzi and Ingram, 2005). We have integrated OT actions in the amygdala with oestrogen effects there and its known neuroanatomy to formulate a four-gene micronet theory that explains certain changes in social recognition in mice.

The involvement of OT in social recognition was initially demonstrated through pharmacological manipulations showing that administration of low levels of OT facilitates social recognition, while OT antagonists block it (Popik and Vetulani, 1991; Popik et al., 1992, 1996). Later, studies with genetically modified mice showed that both males (Ferguson et al., 2000) and females (Choleris et al., 2003) OTKO mice have a complete deficit in social recognition even when tested with the more sensitive choice test paradigm (Choleris et al., 2006). OTRKO mice, too, are impaired in social recognition, further confirming the critical involvement of this system (Takayanagi et al., 2005). Further studies then pointed at the medial amygdala as the site of action of OT and OTR in the regulation of social recognition. The deficit of the OTKO male mice can be rescued by infusion of OT in the medial amygdala, whereas infusion of an OT antagonist inhibits social recognition in wild-type males (Ferguson et al., 2001). Similarly, wild-type females that receive an antisense oligonucleotide targeting the mRNA of the OTR gene in the medial amygdala become completely impaired in social recognition (Choleris, 2007). Like for the α-ERKO and β-ERKO mice, even in the OTKO mice impaired social recognition is reflected in impaired capability of recognizing and avoiding parasitized conspecifics (Kavaliers et al., 2004). As well, the OTKO mice are impaired in utilizing other mice as a source of information in mate choices and parasite avoidance (Kavaliers et al., 2004).

OT involvement in social disorders has been demonstrated. Low OT plasma levels are observed in autistic patient populations (Modhal et al., 1998; Green et al., 2001) where altered OT production from its pro-hormone precursor is shown (Green et al., 2001). Furthermore, in initial clinical trials intravenous infusion of OT ameliorated behavioural symptoms of autism in adult patients (Hollander et al., 2003). Alterations in the OT system are observed also in individuals affected with schizophrenia (Mai et al., 1993; Bernstein et al., 1998; Feifel and Reza, 1999) and depression (Bernstein et al., 1998; Uvnäs-Moberg et al., 1999).

The specific impairment in social recognition of the α-ERKO, β-ERKO and OTKO mice prompted the proposal of a four-gene micronet model to explain the action of oestrogens on the oxytocinergic system in the regulation of this behaviour. In this model, ERβ regulates the production of OT in the PVN, while ERα controls the transcription of the gene for OTR in the medial amygdala which, in turn, receives and processes olfactory input of social relevance from the main and accessory olfactory systems (Dulac and Torello, 2003; Johnston, 2003). This model is supported by molecular biology studies and fully explains the behaviour of the KO mice. First, ERβ is highly expressed in the mouse PVN where ERα is almost absent (Mitra et al., 2003), and directly regulates the production of OT (Patisaul et al., 2003). Accordingly, oestrogen regulation of OT is inhibited in β-ERKO mice (Nomura et al., 2002). Second, ERα is highly expressed in the amygdala (Mitra et al., 2003) where it is necessary for the induction of OTR (Young et al., 1998).

This model explains the behavioural results of the α-ERKO and β-ERKO mice in the more-sensitive choice test paradigm (Choleris et al., 2006). The essentiality of ERα for OTR production in the amygdala (Young et al., 1998) explains the complete impairment of the α-ERKO mice, while the partial impairment of the β-ERKO mice (Choleris et al., 2006) can be explained by an ERβ-mediated upregulation of existing baseline production of OT in the PVN (Mitra et al., 2003). Accordingly, baseline OT levels and mRNA of the OT gene in the PVN of β-ERKO mice are normal, but they fail to respond to stimulation by oestrogens (Nomura et al., 2002). The baseline levels of OT likely allow for a certain degree of social discrimination in β-ERKO mice, which in normal mice can be enhanced following oestrogens/ERβ-mediated increase in OT production.

McCarthy et al. (1996) have reported that OT has anxiolytic properties if and only if oestrogens are circulating in an adequately high concentration. This requirement for oestrogens presumably is due to the strong influence of oestrogens on OTR gene transcription (Young et al., 1998). In fact, in females, blocking OTR activity in the brain increases anxiety-like behaviours in a manner that depends on the hormonal state of the female (Neumann et al., 2000a, b). In the male, testosterone-dependent sexual activity can be followed by the reduction of anxiety due, at least in part, to the release of OT within the brain (Waldherr and Neumann, 2007, in press).

While OT and its functional relations with sex hormones and their receptors have been emphasized in this section, there is no need to minimize the role played by VP. Friendly partner preference formation among voles can be substantially increased by using viral vector technology to cause expression of the VP V1a receptor in the ventral septum and ventral forebrain (Lim et al., 2004). Further, Bielsky et al. (2005) used transgenic mice in which a null mutation in the V1a receptor gene was followed by a re-expression of the V1a receptor specifically in the septum. This re-expression rescued social recognition from the impairment seen during tests with the V1a receptor knockout. In contrast, blocking V1a receptors bilaterally in the amygdala did not significantly reduce social recognition, even while blocking them in the septum did. Thus, VP and its V1a receptor play a role in social recognition, but the neuroanatomical region most involved is the septum, not the amygdala.

Generalized CNS arousal mechanisms related to fear

It has been hypothesized that a large number of ascending and descending neuronal systems involving the expression of more than 120 genes are involved in the adaptive regulation of CNS arousal (Pfaff, 2006). Some of initial need states leading to arousal, such as hunger, are quite specific. However, based on results of a meta-analysis using the mathematical statistical technique of principal components analysis, we have argued that there is a generalized arousal component, an "*urarousal*", that can account for as much as one-third of arousal-related behaviours (Garey et al., 2003). Of special interest for the present discussion are the effects of generalized arousal neurotransmitters in the amygdala.

Inputs to the amygdala from ascending systems that drive generalized arousal might be important for social anxiety, related to the recognition mechanisms just reviewed above, because this same brain region implicated in social recognition, the amygdala, is crucial for producing the emotion of fear. If signals from conditioned stimuli for fear do not reach the amygdala, then conditioned fearful responses do not occur (reviewed in LeDoux, 2000; Rodrigues et al., 2004; Schafe et al., 2005). Likewise, if outputs from the amygdala are suppressed, for example under the influence of the prefrontal cortex, then fear is reduced. In fact, neuropharmacological approaches to the suppression of amygdaloid facilitation of fear is important not only for syndromes such as post-traumatic stress disorder but also, according to our theorizing below, to reducing the social anxiety of autism (Ressler et al., 2004; Davis, 2005). The importance of the amygdala for fear, established in laboratory rodents, holds true for higher primates, including humans (Kalin et al., 2004; Phelps and LeDoux, 2005; Paton et al., 2006). What are the relations of these mechanisms to generalized arousal?

Frightening emotions and emotional memories will not operate correctly to raise fear in a biologically adaptive fashion if the entire CNS has not been aroused. James McGaugh and his colleagues have reported (Roozendaal et al., 2004, 2006; McIntyre and McGaugh, 2005) that the proper operation of amygdaloid mechanisms related to fear depend on synaptic inputs releasing the arousal-related transmitters norepinephrine and dopamine. For example, McGaugh and his colleagues reported that they trained rats in a task in which the animals had to avoid returning to a place where their feet had been shocked. Infusing a dopamine receptor antagonist into the lateral amygdala prevented peak performance of fear learning. Conversely, infusing dopamine itself or, for that matter, norepinephrine, into the amygdala

enhanced retention of the learned fear response. Even additional shocks between training and testing — as would arouse the animal — increase subsequent fear responses. Thus, animals with low levels of generalized arousal are less likely to show high levels of learned fear responses.

Another arousal-supporting neurotransmitter, serotonin is also involved in the production of anxiety and fear. Serotonin-containing fibres reach the amygdala through long axonal projections from the median and dorsal raphe nuclei of the midbrain. Some of the most exciting work on genetic contributions to fear and anxiety has dealt with the serotonin transporter (5-HTT), the molecule responsible for the re-uptake of serotonin from its synaptic cleft. It is now widely recognized the gene encoding 5-HTT contains a 44 base pair sequence that in some individuals is inserted — producing a long allele that has high transcriptional activity — and in other individuals it is deleted — producing a short allele that has less transcriptional activity. In cell cultures, this translates into a twofold greater rate of re-uptake when the long allele is present. What does this mean for anxiety and fear and the amygdala? Three lines of evidence gathered so far show its importance. First, human subjects with one or two copies of the short allele exhibit greater amygdala neuronal activity as assessed by functional magnetic resonance imaging (fMRI) responses to pictures of frightened or angry faces (Hariri et al., 2002). Second, subjects with a short allele show stronger coupling between amygdala and prefrontal cortex fMRI responses to aversive pictures (Heinz et al., 2005). Since this part of the cortex can act to suppress amygdaloid output, their increased correlation is of undoubted significance and the mechanism remains to be discovered. Third, as expected, patients with one or two copies of the short allele actually showed increased levels of anxiety-related traits, state anxiety and enhanced activation in the right amygdala to anxiety provocation (Furmark et al., 2004).

The biophysical mechanisms by which dopamine, norepinephrine and serotonin influence the forebrain so as to affect fear learning remain to be worked out. However, some early clues can be derived from the electrophsiological results of Anthony Grace and his colleagues. In vivo intracellular recordings from neurons in the lateral amygdala revealed that stimuli in the prefrontal cortex, a brain region that inhibits the amygdala's management of fear, suppressed amygdala neuronal activity (Rosenkranz et al., 2003). Dopamine receptor activation by apomorphine attenuated these prefrontal cortical effects (Rosenkranz and Grace, 2001) and thus would disinhibit amygdala-driven fear responses. Dopamine has this effect, in part, by increasing neuronal input resistance (opposing the decrease exerted by prefrontal cortex) through the occupation of DAD2 receptors and by suppressing prefrontal cortex-evoked postsynaptic potentials through the occupation of DAD1 receptors (Rosenkranz and Grace, 2002). Other mechanisms for dopamine effects may be in play, and norepinephrine and serotonin effects remain to be analysed.

Actions of norepinephrine, ascending from the lower brainstem, on PVN neurons also are interesting and complex (Daftary et al., 2000; Banihashemi and Rinaman, 2006; Khan et al., 2006; Balcita-Pedicino and Rinaman, 2007). For example, it appears that the activation of PVN VP-expressing neurons by immune challenges is mediated by alpha-1 adrenergic receptors (Xu et al., 2005), whereas different biophysical actions of norepinephrine on PVN neurons depend on different receptor subtypes, α and β (Daftary et al., 2000).

What about neurochemicals such as opioid peptides *that decrease* arousal? For relevance to social anxiety, the most important sub-nuclei of the amygdala are the lateral and the basolateral amygdaloid areas. Neurons in these areas express high levels of receptors for all three major opioid subtypes, mu, delta and kappa receptors (Mansour et al., 1987). Ligands for the mu receptor, endomorphins, are expressed in the amygdala and have been connected to various aspects of social behaviours in experimental animals (Fichna et al., 2007). Further, the opioid-receptor-like receptor, the nociceptin receptor, is expressed in the amygdala, as is its ligand nociceptin (Sinchak et al., 2006). This finding brings thinking about opioid systems into direct registration with the reproductive hormones and nuclear receptors we

have considered in the four-gene micronet, because oestrogen administration to ovariectomized rats increased gene expression for nociceptin in the amygdala (Sinchak et al., 2006). Moreover, learned fear, for which the amygdala is essential, is significantly reduced by the mu opioid antagonist Clocinnamox, but only in the presence of oestrogens (Devidze et al., unpublished data). That is, oestrogens have a permissive action on the mu opioid antagonist effect on fear. Can opioid actions also be brought into registration with OT effects on social recognition and social anxiety? This is harder to say, because that is a nascent field of research and the relations between opioid actions and OT actions appear to depend on the target tissue: cooperative in the nucleus accumbens (Gu and Yu, 2007), but antagonistic in smooth muscles (Nacitarhan et al., 2007). More generally, opioids are released during bouts of social contact, they mediate social learning of food preference (Moles et al., 1999) and also have been reported to diminish the reaction to social separation (Mayes, 2006). Consistently, mu receptor KO mice show impaired social attachment (Moles et al., 2004).

Thus, even as neurochemicals that increase levels of arousal could heighten activity in the amygdala related to fear and anxiety, other neurochemicals such as opioid peptides may have effects in the opposite direction.

Hyperarousal fostering social anxiety

There is little doubt that prolonged high levels of arousal are aversive. The Yerkes–Dodson law, supported by a century of research, states that task performance will consequently be reduced. This would be expected to include "social tasks" in which appropriate behaviour with another individual is required.

There are at least three levels of mechanisms to discuss in dealing with the connection between hyperarousal and social anxiety. First, it is easy to think of long-lasting high levels of activity in the ascending arousal systems including those mentioned above as causing a socially anxious state. Second and more complex are the possible roles of the neuropeptides OT and VP themselves. They both affect the autonomic nervous systems, are both connected with the regulation of fluid balance in the body and are both involved in the regulation of smooth muscle contraction. The simplest formulation is to state that OT is more concerned with autonomic responses associated with reproduction in safe situations (lactation, delivery of babies), while VP is more important with emergency responses to threatening situations (dehydration, haemorrhage). Both of these physiological levels of hypothesis will benefit from comparisons among high-anxiety and low-anxiety lines of rats (Landgraf and Wigger, 2002, 2003). Third is the most psychological level of exploration. It considers a "mismatch hypothesis" that social anxiety results in part from a feeling of lack of preparation for the social encounter. If feeling adequately prepared and if OT and VP levels are optimal, then we feel supported and social anxiety does not occur. If instead we are not adequately prepared and/or if OT and VP levels are not optimal, then as a result we certainly will be hyperaroused and the anticipation of the social event, whatever it may be, will be anxiogenic.

Social anxiety fostering autism

We theorize that imbalances among the levels of expression of certain genes in neurons within the amygdala or among expression of genes in CNS arousal pathways lead to the appearance of autism spectrum disorders. Already, the notion of strong amygdaloid involvement receives strong support from the literature. Ralph Adolphs and his colleagues (Spezio et al., 2007) have found that substantial damage to the amygdala reduces eye contact during conversations, following up earlier work (Adolphs et al., 2005) during which destruction of the amygdala in a human was found to have damaged the ability to respond to fearful expressions of others in a normal way. Conversely, scientists working with Andreas Meyer-Lindenberg (Kirsch et al., 2005) reported that human amygdaloid function is modulated by OT, in that fear-inducing visual stimuli did not activate the amygdala in human subjects given

intranasal applications of OT. Thus, we hypothesize that in the amygdala OT action will reduce the potential for autism, while excess stimulation from ascending arousal pathways — typical perhaps with Asperbergers patients — will increase the likelihood of autism. Consistently, autistic children have enlarged amygdalar volume (Schumann et al., 2004).

The possible role of ascending arousal systems, such as dopamine, norepinephrine and serotonin, influencing the amygdala in such a manner as to increase social anxiety has received some support. All three of these neurotransmitters are imbalanced in autism (Penn, 2006). Likewise, there are a few studies showing opioid dysfunction in autistics, also summarized by Penn (2006).

What about sex differences in autism? Approximately 80% of diagnosed autism patients are male, and about 90% of high-end autism spectrum disorder patients are male. We hypothesize that in females high levels of oestrogens dampen down the effects of ascending arousal systems in such a manner as to reduce social anxiety. McCarthy's (1996) evidence quoted earlier supports this idea. Accordingly, the synaptic organization in the medial amygdala of rats is sexually dimorphic and lateralized before puberty, with females having lower left amygdala excitatory synaptic activity than males. This is proposed to provide a sexually dimorphic substrate for hormonal effects on adult social behaviour (Cooke and Woolley, 2005). Is there not enough oestrogen? If these males have high enough testosterone levels, is there not enough aromatase enzyme activity to convert that testosterone into estradiol? These questions remain unanswered. Further complexity comes from considering progesterone. Females late in pregnancy with high levels of oestrogens and progestins are less anxious, showing less responsiveness to stress (Douglas et al., 1998, 2003). These behavioural effects might be mediated by transcriptional actions of the liganded progesterone receptor but might also be due to the actions of reduced progesterone metabolites that allosterically enhance activity of the GABA receptor. It is possible that all of these hormonal actions synergistically reduce the capacity for social anxiety in the female compared to the male and, as a result, enjoy a lower incidence of autism.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 25

Functional magnetic resonance imaging and the neurobiology of vasopressin and oxytocin

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Abstract: Functional magnetic resonance imaging is used to assess the roles of vasopressin (AVP) in aggressive motivation and oxytocin (OXT) in maternal behaviour. In the case of aggression, male rats are triggered to show the autonomic signs of impending attack behaviour by simulating a resident/intruder conflict in the bore of the magnet during the imaging session. Intraventricular injection of AVP alone causes robust changes in brain activity mirroring many areas selective for AVP receptor binding and overlapped with many of the same areas activated during aggression. Pretreatment with an orally active AVP V1a receptor antagonist blocks aggression in bench-top studies and suppressed the pattern of brain activation caused by the intruder or administration of AVP. These findings using imaging corroborate and extended our understanding of the neural circuitry of aggression and the role of AVP neurotransmission in agonistic motivation. In the case of maternal behaviour, primiparous dams are imaged during nursing with and without OXT receptor blockade. Suckling alone activates many cortical and subcortical areas. Intracerebroventricular injection of OXT stimulates brain activity in areas selective to OXT receptor binding and overlap with many of the same areas activated during pup suckling. Pretreatment with OXT receptor antagonist suppresses the pattern of brain activation caused by suckling or administration of OXT. The data suggest that OXT may strengthen mother–infant bond formation partly by acting through brain areas involved in regulating olfactory discrimination, emotions and reward.

Keywords: aggression; maternal behaviour; BOLD imaging; serotonin; pup suckling; vasopressin receptor antagonist; SRX251

Introduction

Vasopressin (AVP) and oxytocin (OXT) comprise a phylogenetically old super family of chemical signals in both vertebrates and invertebrates. The conservation and dispersion of AVP and OXT signalling systems across the animal kingdom attests to their functional significance in evolution. In addition to their function in physiological homoeostasis, these neuropeptides evolved a role in social behaviours related to aggression and affiliation. AVP has a demonstrated role in aggression enhancing agonistic behaviour in amphibians, fish, birds and mammals, including humans. In contrast, OXT is a key hormone enabling milk let-down during breastfeeding and the enhancement of affiliative behaviours associated with caring for young. While both neuropeptides function as neurohormones released from the posterior pituitary gland into the general circulation, their effects

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DOI: 10.1016/S0079-6123(08)00425-1

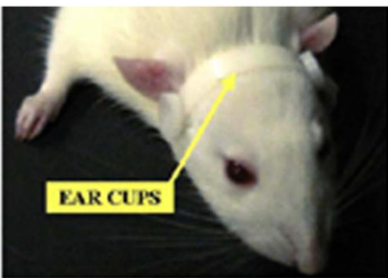
on behaviour are achieved by direct neurochemical signalling in the central nervous system. Precisely how and where these neuropeptides act to affect such important behaviours as aggression and affiliation are not entirely clear. With the advent of new non-invasive imaging techniques like functional magnetic resonance imaging (fMRI) it is possible to gain new insights into the neurobiology of these neuropeptides. To this end, fMRI with 3D computational analysis in conscious rats was used to study the role AVP in aggressive motivation and the role of OXT in pup suckling and maternal behaviour.

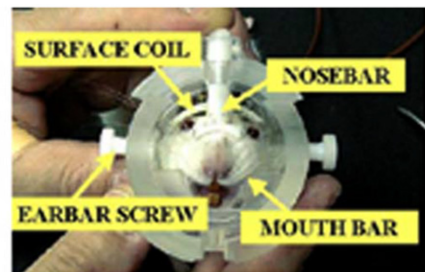
Functional MRI with ultra-high field animal scanners ($\geq$4.7 T) provide exceptional temporal and spatial resolution making it possible to map in seconds functionally relevant neural networks activated by a variety of environmental and chemical stimuli (Ferris et al., 2001; Tenney et al., 2004; Brevard et al., 2006a, b). Increased neuronal activity is accompanied by an increase in metabolism concomitant with changes in cerebral blood flow and blood volume to the area of elevated neural activity. Blood oxygen level-dependent (BOLD) fMRI is a technique sensitive to the oxygenation status of haemoglobin (Ogawa et al., 1990). While fMRI has neither the cellular spatial resolution of immunostaining, nor the millisecond temporal resolution of electrophysiology, it does show synchronized changes in neuronal activity across multiple brain areas, providing a unique insight into functional neuroanatomical circuits coordinating the thoughts, memories and emotions for particular behavioural states. This chapter presents a discussion on the technology and methods for performing imaging studies on awake male rats responding to aggression-provoking stimuli and awake lactating dams responding to pup suckling. These unique experimental models enable two questions: (1) What is the role of centrally released AVP in aggressive motivation? (2) What role does OXT play in suckling-induced brain activation?

Imaging conscious animals

Animals are acquired and cared for in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 85-23, Revised 1985) and adhere to the National Institutes of Health and the American Association for Laboratory Animal Science guidelines. The protocols used in these studies were in compliance with the regulations of the Institutional Animal Care and Use Committee at the University Massachusetts Medical School.

Studies are performed with a multi-concentric dual-coil, small animal restrainer (Insight Neuroimaging Systems, LLC, Worcester, MA). The basic configuration used for rat MR imaging is shown Fig. 1. The BOLD fMRI signal is detected through the manipulation of precessing protons in the various brain tissue compartments when a subject is placed within an external magnetic field (B_0 field provided by the MR scanner). Two radiofrequency (Rf) coils, one for transmission and one for reception, facilitate the manipulation and detection of changes in proton resonant frequency, as an indirect measurement of the fMRI signal in vivo. Prior to imaging studies, animals are anaesthetized with 2–4% isoflurane. A topical anaesthetic of 10% lidocaine cream is applied to the skin and soft tissue around the ear canals and over the bridge of the nose. A plastic semicircular headpiece with blunted ear supports that fit into the ear canals are positioned over the ears (Fig. 1). The head is placed into a cylindrical head holder with the animal's canines secured over a bite bar and ears positioned inside the head holder with adjustable screws fitted into lateral sleeves (Fig. 1). An adjustable Rf surface coil built into the head holder is pressed firmly on the head and locked into place. This Rf coil facilitates the detection of changes in resonant frequency signals from brain tissues and works in conjunction with the overlying volume Rf coil that transmits Rf pulses into the brain (Ludwig et al., 2004). The body of the animal is placed into a body restrainer. The body restrainer "floats" down the centre of the chassis connecting at the front and rear endplates and buffered by rubber gaskets. The headpiece locks into a mounting post on the front of the chassis. This design isolates all of the body movements from the head restrainer and minimizes motion artefact. Once the animal is positioned in the body





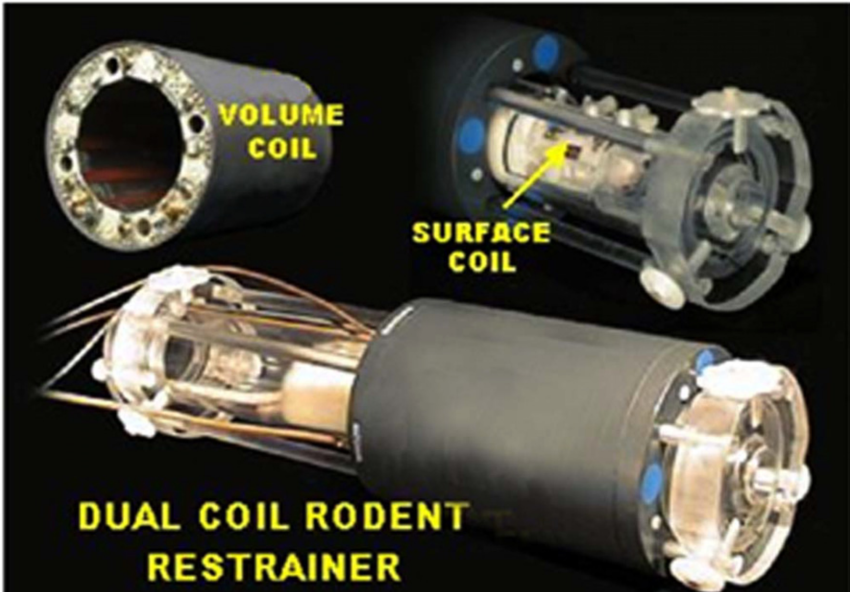


Fig. 1. Imaging set-up. The top panels shown are the different components of the head restrainer and the positioning of the rat. The bottom panel shows the complete restraining device with Rf electronics. Adapted with permission from Ludwig et al. (2004). (See Color Plate 25.1 in color plate section.)

holder, a volume Rf coil is slid over the head restrainer and locked into position.

Prior to imaging, animals are routinely acclimated to the restrainer and the imaging protocol. Animals are anaesthetized with isoflurane as described above. When fully conscious, the restraining unit is placed into a black opaque tube "mock scanner" with a tape-recording of an MRI pulse sequence for 90 min in order to simulate the bore of the magnet and an imaging protocol. This procedure is repeated every other day for 4 days. With this procedure, rats show a significant decline in respiration, heart rate, motor movements and plasma corticosterone when comparing the first to the last acclimation periods (King et al., 2005). The reduction in autonomic and somatic measures of arousal and stress improve the signal resolution and quality of the MR images. After this acclimation procedure females are pair housed with male breeders for mating.

Experiments are conducted in a Bruker Biospec 4.7-T/40-cm horizontal magnet (Oxford Instrument, Oxford, UK) equipped with a Biospec Bruker console (Bruker, Billerica, MA, USA). Functional images are acquired using a T2 weighted multi-slice fast spin echo sequence. A single data acquisition acquires twelve, 1.2 mm slices in 6–8 s. The imaging sessions usually consist of 3–5 min of baseline data followed by 5–10 min of stimulation data. At the end of each imaging session a high-resolution

anatomical dataset that exactly matches the geometry of the functional scan is collected.

Anatomy images for each subject are registered to a segmented rat brain atlas as previously described (Ferris et al., 2005). The fully segmented rat brain atlas has the potential to delineate and analyse more than 1200 distinct anatomical volumes within the brain. Because the in-plane spatial resolution of our functional scans is 486 μm^2 with a depth of 1200 μm, many small brain areas (e.g. nucleus of the lateral olfactory tract) can not be resolved; or, if they could be resolved they would be represented by one or two voxels only (e.g. arcuate nucleus of the hypothalamus). Consequently, small detailed regions are not included in the analysis or are grouped into larger ''minor volumes'' of similar anatomical classification. For example, in the aggression imaging study the basal nucleus of the amygdala is listed as a minor volume. This area is a composition of the basomedial anterior part, basomedial posterior part, basolateral anterior part and basolateral posterior part with a composite voxel size of 54. In addition, we grouped brain areas into ''major volumes'' (e.g. amygdala, hippocampus, hypothalamus, cerebrum). The volume of activation (number of significant voxels) can be visualized in these 3D major and minor anatomical groupings (Fig. 4). We also combined minor volumes to form functional neuroanatomical pathways as shown in Fig. 5.

There are certain limitations and complications to imaging awake animals. First and foremost is the restraint of the head, without which it would be impossible to collect a clean image. Head restraint precludes the study of many behaviours that require a consummatory act, as the immobilization alone may prevent the motor response that defines the behaviour. Offensive aggression as measured by the latency to bite and number of bites toward a conspecific is a case in point. However, internal states of arousal and motivation like, hunger, fear and aggressive intent are fertile areas of investigation using fMRI and awake animals.

The stress of head restraint and restricted body movement is compounded by the noise and duration of the imaging protocols. Consequently, sensory- or drug-induced changes in MR signal in awake animals can occur against a backdrop of heightened arousal and stress — conditions that can affect data interpretation. To address these problems, protocols have been developed for acclimating animals to the environment of the MR scanner and imaging procedure leading to a reduction in stress hormones levels and measures of autonomic activity regulated by the sympathetic nervous system (Zhang et al., 2000; King et al., 2005). Acclimation protocols have been used to prepare awake animals for a range of behavioural, neurological and pharmacological imaging studies, including sexual arousal in monkeys (Ferris et al., 2004), generalized seizures in rats and monkeys (Tenney et al., 2003, 2004), and exposure to psychostimulants like cocaine (Febo et al., 2004, 2005; Ferris et al., 2005), nicotine (Skoubis et al., 2006) and apomorphine (Zhang et al., 2000; Chin et al., 2006). Habituation to the scanning session is achieved by putting subjects through several simulated imaging studies. It is recognized that some stress is still likely associated with the imaging procedure. For example, piloerection in resident males as discussed below usually occurs within 20 s of introduction of the intruder male in a home cage test, while the response is delayed for up to 60 s in the imaging environment. This delay may be due to the added complication of head restrain and stress in this experimental paradigm. Nonetheless, the piloerection occurs reliably and is highly correlated with the peak BOLD response in many brain areas.

Imaging aggressive motivation and the role of vasopressin neurotransmission

There is a general consensus that AVP functions to facilitate aggressive behaviour across multiple species (Ferris, 2005). Microinjections of AVP into the hypothalamus or amygdala and cerebrointraventricular administration in rodents leads to enhanced aggression while administration of a selective linear V1a antagonist, Manning compound [1-β-mercapto-β,β-cyclopentamethylene propionic acid 2-[0-(methyl) tyrosine] arginine vasopressin, blocks aggressive behaviour (Ferris and Potegal,

1988; Potegal and Ferris, 1990; Young et al., 1997; Harrison et al., 2000; Caldwell and Albers, 2004). In humans indices of aggressivity correlate with high concentrations of AVP in cerebrospinal fluid (Haller et al., 1996; Coccaro et al., 1998). Intranasal AVP stimulates agonistic facial motor patterns in response to faces of unfamiliar men and biases male subjects to interpret neutral facial expressions as potentially aggressive (Thompson et al., 2004, 2006).

Inappropriate aggressive behaviour is closely correlated with changes in the neurobiology of the AVP system. Peripubertal hamsters socially subjugated by dominant male hamsters show altered AVP immunoreactivity in the hypothalamus as young adults and heightened aggression towards smaller conspecifics as compared to non-subjugated littermates (Delville et al., 1998). Newborn rat pups stress by maternal separation show increased AVP fibres in the lateral hypothalamus as adults and heightened aggression as compared to littermate controls (Veenema et al., 2006). Treating adolescent hamsters with anabolic steroids increases the density of AVP immunoreactive fibres, V1a receptor and neuropeptide content in the hypothalamus and enhances AVP-mediated aggression in adulthood (Harrison et al., 2000; DeLeon et al., 2002). Peripubertal hamsters exposed to cocaine develop a highly aggressive phenotype as adults and enhanced released of AVP in the hypothalamus (Jackson et al., 2005). The development of dominant/subordinate relationships in hamsters causes a reduction in AVP levels in the hypothalamus in submissive partners (Ferris et al., 1989) while dominant partners show higher levels of V1a binding in the hypothalamus (Cooper et al., 2005). Mice with distinct behavioural phenotypes of high and low aggressivity, show correspondingly high and low levels of AVP receptor density and fibre immunostaining in bed nucleus of the stria terminalis and lateral septum (Bester-Meredith et al., 1999). When high aggressive phenotypes are cross-fostered with low aggressive parents they show a reduction in aggression in a resident–intruder paradigm and lower levels of AVP in the bed nucleus as compared to their unfostered siblings (Bester-Meredith and Marker, 2001).

Method for imaging aggressive motivation

Adult male and female Long-Evans rats are housed as male/female pairs and maintained on a 12:12 light:dark cycle (lights on at 7:00 h) and provided food and water ad libitum. The minimum duration of cohabitation between male/female pairs before testing is 2–3 weeks. Prior to housing, all females undergo tubal ligation to prevent pregnancy. On the day of an imaging session, male residents are tested for aggressive motivation by placing a novel, adult male intruder into their home cage for 5 min. The resident is timed for the onset of piloerection of the fur along the lower midline back (Fig. 2). The average time to piloerection in the home cage environment is approximately 18 s. Following this home cage test, resident males are secured in the restrainer used for imaging as described below. Once positioned in the scanner, the resident is exposed to his female cage mate alone or his mate plus a novel intruder. During the imaging session it is possible to observe the back of the restrained resident (Fig. 2) and time the onset of piloerection. The average time to piloerection in the magnet is approximately 60 s.

Resident males are given an oral dose of SRX251 (Azevan Pharmaceuticals, Bethlehem, PA). SRX251 is a highly selective, orally active AVP V1a receptor antagonist that can cross the blood–brain barrier (Ferris et al., 2006). It was determined that a 5 mg/kg dose of SRX251 blocked piloerection in the home cage environment; consequently, on the day of imaging, male residents are given an oral dose of 5 mg/kg SRX251 and approximately 90–120 min later imaged. During the imaging session they were presented with a novel male intruder in the presence of their female cage mate.

To test if the serenic activity of SRX251 is specific to aggression and not generalized to all highly emotional stimuli associated with autonomic arousal, drug treated male residents were tested for sexual motivation in the presence of a novel female. Novel females are ovariectomized and following recovery, treated with oestrogen/progesterone to induce oestrus, sexual receptivity and lordosis in response to tactile stimulation. In a home cage test, the resident female is removed and

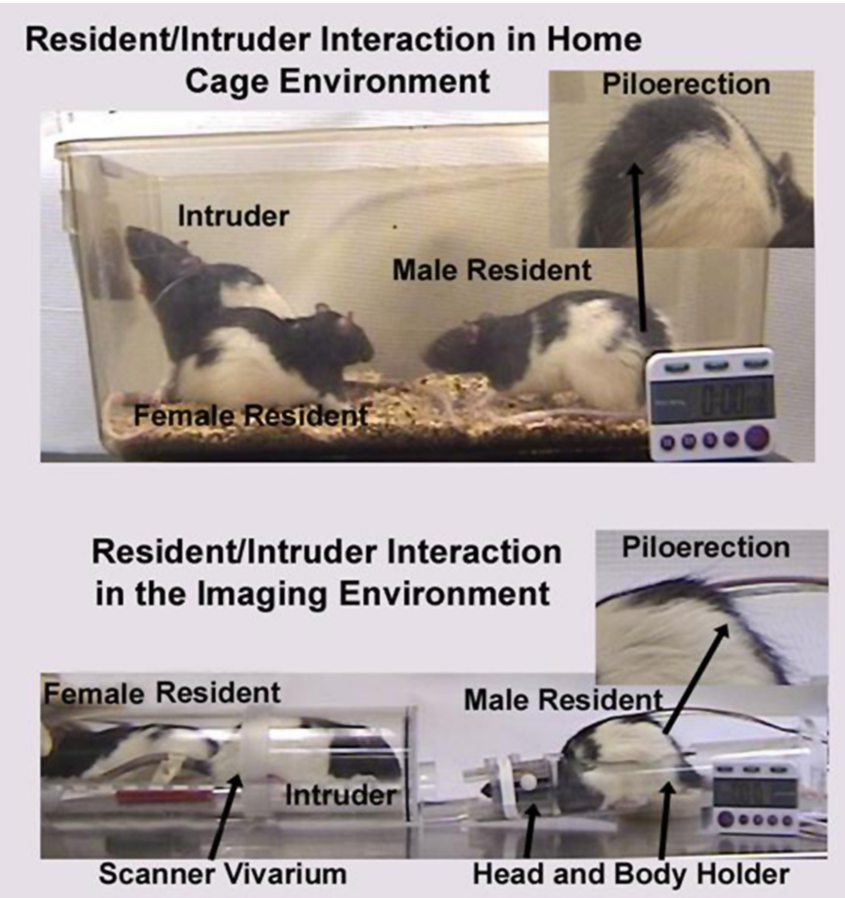


Fig. 2. Piloerection in the home cage and imaging environment. The top photograph shows a male and female resident in their home cage moments after the introduction of a novel adult male intruder. The insert shows a photograph of piloerection along the midline back of the male resident. The bottom photograph shows a male resident with his head secured in the animal restrainer used for imaging. The animal's body is unrestrained and the body holder is open at the top to allow visualization of the animal's back. A vivarium designed to fit into the bore of the scanner immediately in front of the male resident is shown housing the resident's female partner and a novel adult male intruder. This presentation of stimulus animals in the vivarium elicits piloerection in the restrained male resident, an autonomic response that can be observed in the bore of the scanner during data acquisition. Adapted with permission from Ferris et al. (2008), manuscript under review, BMC Neuroscience.

replaced with a novel receptive female. Resident males are tested for latency to mount the female 90–120 min following treatment with 5 mg/kg SRX251. The latency to mount and thrust the novel female is ca. 100 s as compared to 90 s for vehicle controls. During the imaging session SRX251 treated males are presented with a novel sexually receptive female.

To assess the direct effect of AVP on brain activating male rats are implanted with a MR compatible intraventricular catheter for the direct injection of AVP (10 ng/10 μl artificial cerebrospinal fluid) into the brain.

Results

Of the 83 brain areas investigated in response to the aggression promoting stimuli of an intruder male, 16 showed a significant increase in the volume of activation over the mate alone (Table 1).

Table 1. Activation of brain areas associated with aggressive motivation

Brain area	Volume of activation	
	Mate alone	Mate + intruder
Retrosplenial cortex	26 (10, 61)	50 (26, 99)**
Orbital cortex	9 (1, 14)	19 (4, 44)*
Auditory cortex	20 (4, 61)	38 (23, 51)*
Somatosensory cortex	114 (16, 266)	221 (141, 392)*
Prelimbic cortex	2 (0, 11)	8 (2, 25)*
CA1 hippocampus	27 (10, 67)	46 (32, 110)*
Dentate gyrus	20 (3, 48)	32 (22, 80)**
Cortical n. amygdala	14 (5, 29)	23 (14, 52)*
Basal n. amygdala	4 (1, 11)	10 (2, 17)**
Medial n. amygdala	1 (0, 6)	3 (0, 9)*
Bed n. stria terminalis	2 (0, 5)	6 (2, 16)*
Lateral post. n. thalamus	2 (0, 9)	8 (2, 11)**
Anterior n. thalamus	3 (0, 6)	6 (2, 12)**
Ventral pallidum	5 (0, 17)	11 (6, 21)*
Lateral hypothalamus	10 (3, 26)	25 (6, 54)*
PVN hypothalamus	1 (0, 2)	3 (1, 5)*

The two columns report the median (min−max) number of voxels activated in male residents when presented with their female cage mate (mate alone) or their cage mate plus a novel adult male intruder (mate + intruder). Male residents ($n = 10$) were tested for each condition in a counterbalanced design and the data analysed using a Wilcoxon Signed-Rank Test. Those areas that showed a significant increase in the number of activated voxels were screened from a database of 83 brain areas and comprise the putative neural circuit of aggressive motivation.

*$p < 0.05$.

**$p < 0.01$.

These 16 areas comprise the putative, distributed neural circuit involved in the control of aggressive motivation. Historically, several of these areas, including the lateral hypothalamus, cortical and medial amygdala and bed nucleus of the stria terminalis, have a key role in the control of aggressive responding. The medial basolateral hypothalamus extending from the mammillary nuclei up through the lateral and anterior hypothalamus has a fundamental role in the organization and initiation of aggressive behaviour in all mammalian species studied to date. The lateral hypothalamus was particularly sensitive showing both a significant increase in the volume of activation and increase in BOLD signal to aggression-provoking stimuli (Fig. 3). The lateral hypothalamus has extensive efferent connections to a majority of the brain areas that constitute the putative neural circuit of aggressive motivation identified with fMRI. Given the pivotal position of the lateral hypothalamus in the neural circuitry of aggression, the a prior hypothesis, routinely used in fMRI studies, predicted activation of this brain area.

Orally administered SRX251 successfully blocks the aggressive motivation of resident males towards male intruders both on the bench-top and during an imaging session. The efficacy of SRX251 as an inhibitor of aggressive motivation, as assessed by fMRI, is characterized by a global suppression of BOLD signal expressed both as a reduction in the volume of activation (Fig. 4) and percent change in BOLD signal in areas that comprise the putative neural circuit of aggressive motivation. This effect of SRX251 appears to be specific because when male residents are challenged with sexually motivating stimuli like the presentation of a novel receptive female, there is an increase in BOLD signal over several brain areas, many of which are not associated with the neural circuit of aggression. Indeed, the activation of the primary olfactory system and mesocorticolimbic system associated with aggressive motivation are dramatically reduced with SRX251 pretreatment, but these effects are not seen in the context of sexual motivation (Fig. 5). One of the more compelling differences in brain activity between

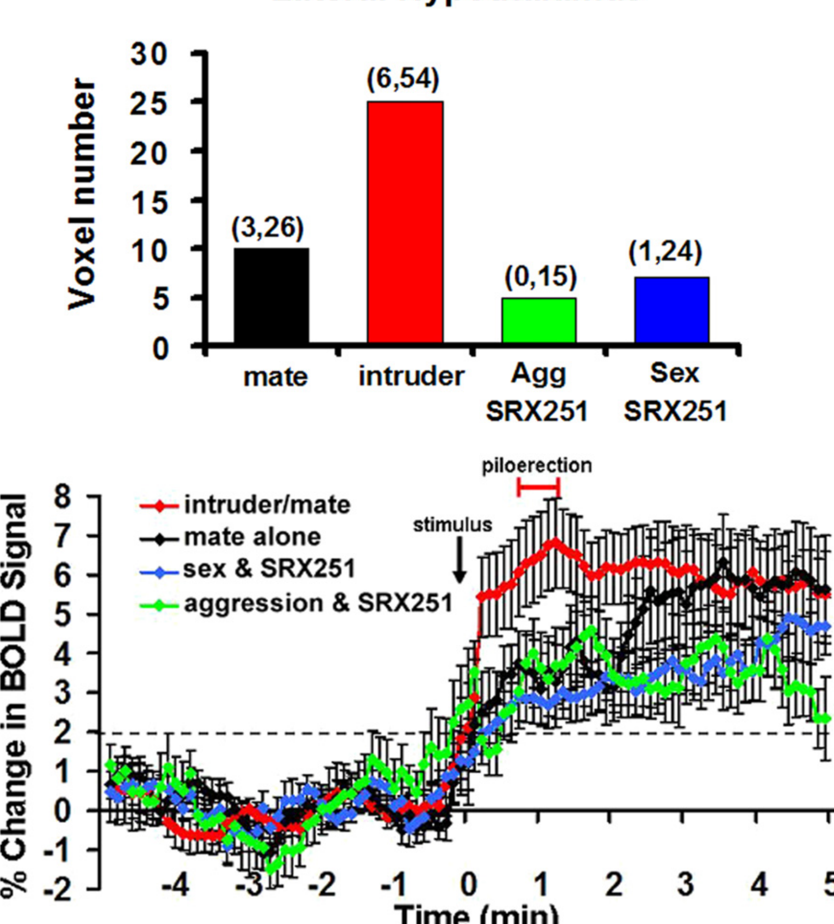


Fig. 3. Lateral hypothalamus and vasopressin-mediated aggression. Above are bar graphs depicting the medium number of voxels (minimum and maximum), i.e. volume of activation, for each imaging condition ($n = 10$ subjects for each condition). Shown below are the changes in BOLD signal intensity following each stimulus presentation (time 0 min). The dashed horizontal line marks the threshold of 2% below which is baseline noise in awake imaging studies. The scale marked piloerection shows the range of time (62 ± 11) for piloerection following introduction of the mate/intruder into the vivarium. The percentage change in BOLD signal intensity at each time point (100 data acquisition over the 10 min scanning period) is the average of 10 male residents for each experimental condition. Vertical lines at each data point denote the standard error of the mean. (See Color Plate 25.3 in color plate section.)

aggressive and sexual motivation following SRX251 treatment was the activation of the dopaminergic pathways originating in the substantia nigra and ventral tegmental area. SRX251 treatment suppresses activity in these areas in response to aggression-provoking stimuli but not to sexual stimuli, which may explain why sexual motivation is spared with V1a receptor antagonism. The substantia nigra shows a significant increase in BOLD signal over time, while the ventral tegmental area and its efferent connections show an ostensible increase in the volume of activation. The nigrostriatal dopaminergic pathway is important in sexual readiness while the mesolimbic dopaminergic pathway affects sexual motivation (Hull, 1995).

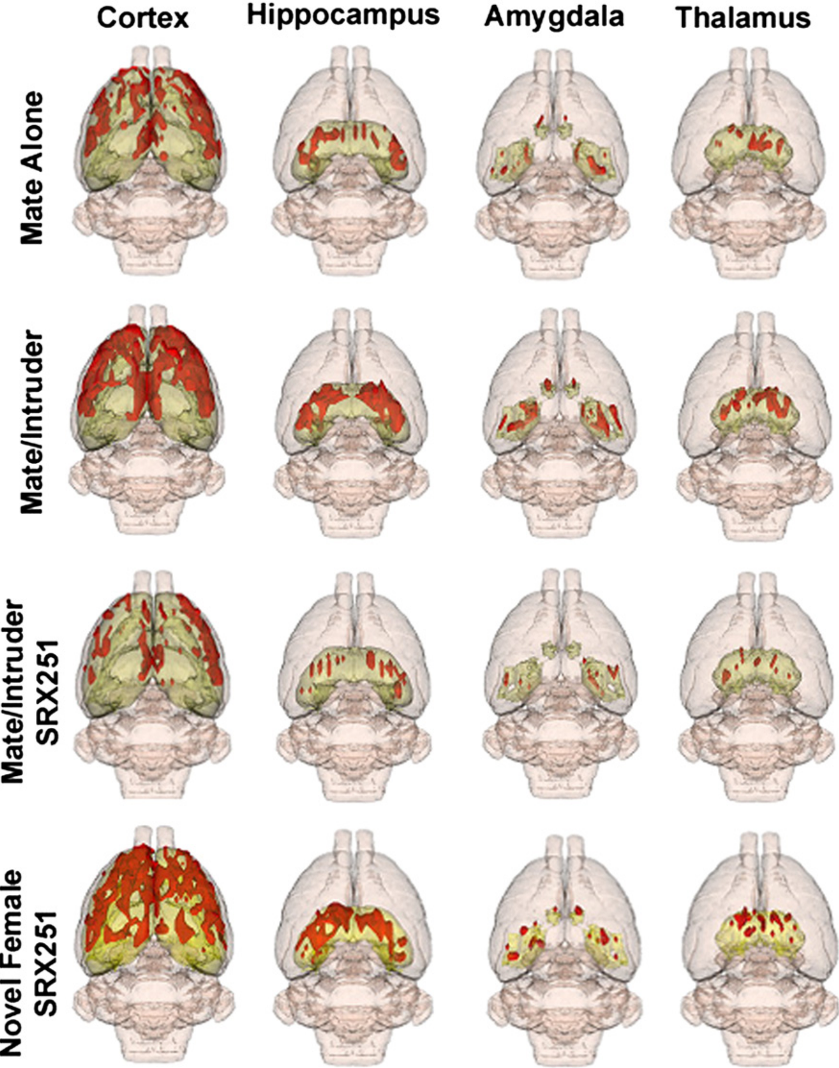


Fig. 4. Three-dimensional representations of BOLD activation. The pictures show translucent shells of the brain viewed from a caudal/dorsal perspective. The red depicts the localization of activated voxels interpolated into a 3D volume of activation for four experimental conditions: mate alone, mate/intruder, pretreatment with SRX251 followed by the aggressive or sexual promoting stimuli. The volumes of activation for each experimental condition are composed of 10 male residents each. Once fully registered and segmented, the statistical responses for each animal are averaged on a voxel-by-voxel bases. Those averaged voxels that are significantly different from baseline and exceed a 2.0% threshold are shown in their appropriate spatial location. The geometric volumes constituting each major area like the hippocampus, i.e. subiculum, dentate gyrus, CA1, CA2, CA3 have been melded into a single volume shown in yellow. Adapted with permission from Ferris et al. (2008), manuscript under review, BMC Neuroscience. (See Color Plate 25.4 in color plate section.)

Intracerebroventicular injection of AVP activates many brain know to localize AVP receptor. Shown in Fig. 6 is an autoradiogram of AVP binding in the medial and lateral, basal hypothalamus (adapted from Barberis et al., 1995). Note the activation of this area following ICV injection of AVP. The volume of activation in this area can be reduced by prior treatment with SRX251.

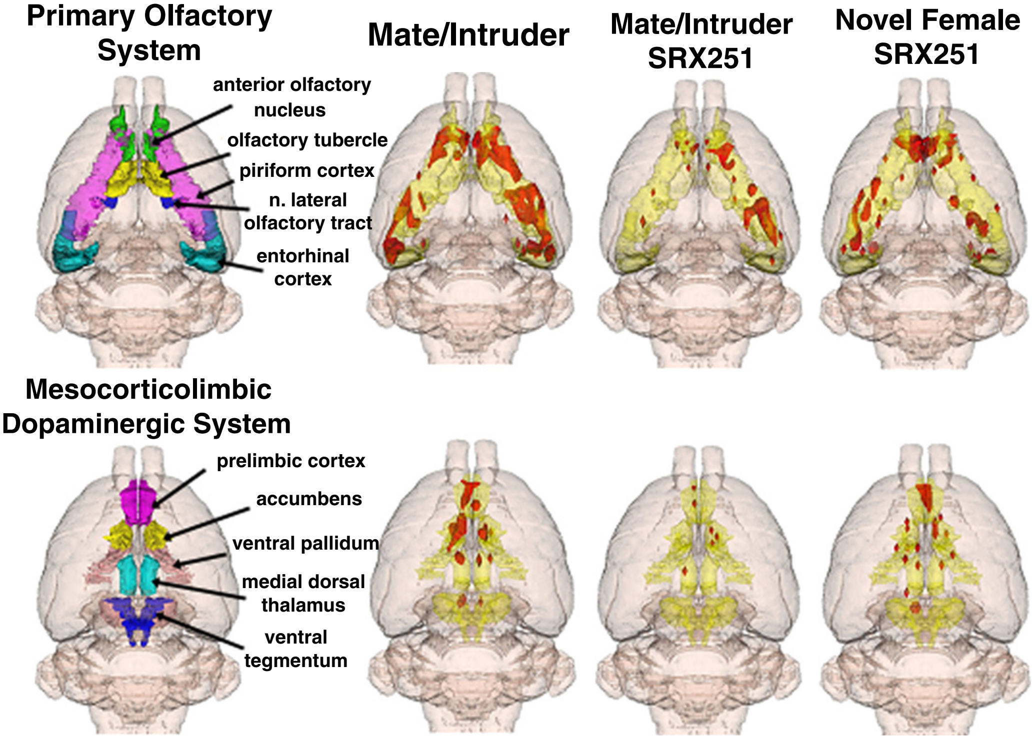


Fig. 5. Selectivity of V1a receptor blockade on aggressive motivation. SRX251 selectively blocks aggressive motivation but not sexual motivation as shown in these 3D models of activity in the olfactory and reward pathways. These 3D volumes of activation are composed of 10 subjects each. Adapted with permission from Ferris et al. (2008), manuscript under review, BMC Neuroscience. (See Color Plate 25.5 in color plate section.)

Conclusion

Treating resident males with the highly specific, orally active, V1a receptor antagonist SRX251 blocks offensive aggression and the peripheral autonomic signs of aggressive motivation. The suppression of brain activity, as assessed through BOLD imaging, is directed towards the neural circuitry identified with the organization and expression of aggression. Brain levels of SRX251 peak within 2–4 h of oral administration and remain elevated for over 8–12 h (Guillon et al., 2006). In a previous study, we showed that the anti-aggressive effects of SRX251 were brain mediated and not due to peripheral blockade of V1a receptors (Ferris et al., 2006). AVP V1a receptor binding is found throughout the brain, particularly in many areas that constitute the neural circuit of aggressive motivation identified with fMRI (Tribollet et al., 1988; Ferris et al., 1993; Insel et al., 1994; Barberis et al., 1995; Young et al., 1999). Specifically, V1a binding is localized to the lateral hypothalamus, BNST, corticomedial amygdala, prelimbic cortex, forebrain cortex, PVN, ventral pallidum, hippocampus and anterior thalamic nuclei. Consequently, treating resident males with SRX251 could suppress aggressive responding by acting at all or some of these brain areas.

Imaging the "nursing" brain and the role of oxytocin neurotransmission

OXT synthesis mainly occurs in neurons of the paraventricular (PVN) and supraoptic nucleus (SON) of the hypothalamus. It is transported to and released from nerve terminals in the posterior

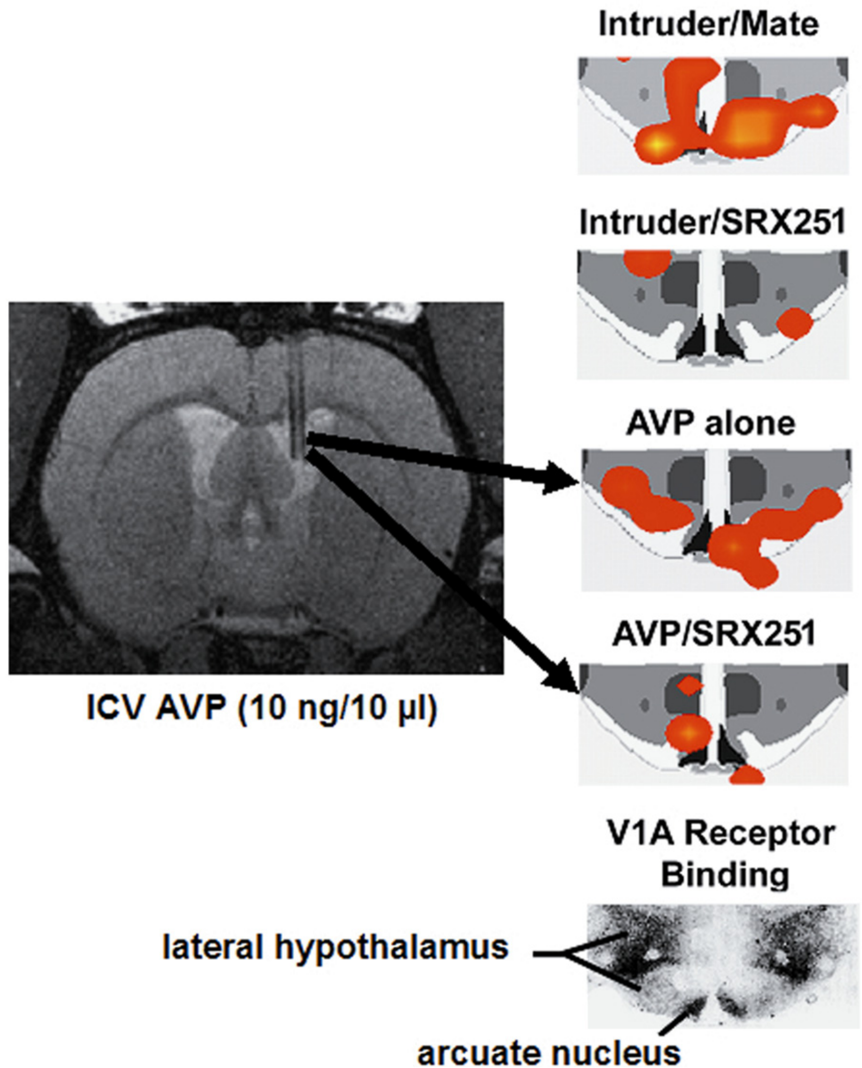


Fig. 6. Intracerebroventricular injection of vasopressin. On the left is a coronal section taken during the anatomical data acquisition of an imaging session showing the placement of the injection cannula into the lateral cerebroventricle. The column of coronal section to the right depicts BOLD activation maps at the rostral-caudal plane of the lateral hypothalamus and arcuate nucleus. The bottom figure in this column is a photomicrograph of an autoradiogram showing localization of specific V1a receptor binding. (See Color Plate 25.6 in color plate section.)

pituitary and various regions of the brain. Suckling stimulates the release of OXT simultaneously into the bloodstream and central nervous system of postpartum rats (Neumann et al., 1993a). Systemically, this neurohormone enhances smooth muscle contractility, which is important for milk 'let-down' during nursing. There is evidence to suggest that the release of OXT in the CNS during parturition initiates the onset of maternal behaviours. Indeed, the expression of maternal behaviours in sheep and rats is delayed following blockade of OXT receptors during parturition (van Leengoed et al., 1987; Levy et al., 1992). OXT release in response to suckling has been measured using microdialysis in the substantia nigra, olfactory bulbs, mediobasal hypothalamus, bed nucleus

of the stria terminalis, medial preoptic area (MPOA) and septum of parturient sheep (Kendrick et al., 1997), as well as in sites of origin, the PVN and SON (Neumann et al., 1993a, b). The release of OXT within the latter two hypothalamic nuclei can be blocked by administration of an OXT receptor antagonist, suggesting a positive feedback mechanism controlling its own release (Neumann et al., 1994). The specific effect of OXT on brain activity following parturition, particularly during breastfeeding remains unclear. Here we tested whether OXT modulates suckling stimulated brain activity in postpartum dams.

Breastfeeding has played a critical role in the survival of mammals. Lactation is an intermittent event in which mothers forage for sustenance while simultaneously converting food into nutrient-rich milk for their young. During the reproductive period defined by lactation, a bond is formed between a mother and her offspring that favours their protection and social development. In rodents, the initiation and maintenance of maternal behaviour progresses through a complex interaction between the endocrine status of the dam prior to and following parturition and the continued interaction and stimulation from pups until

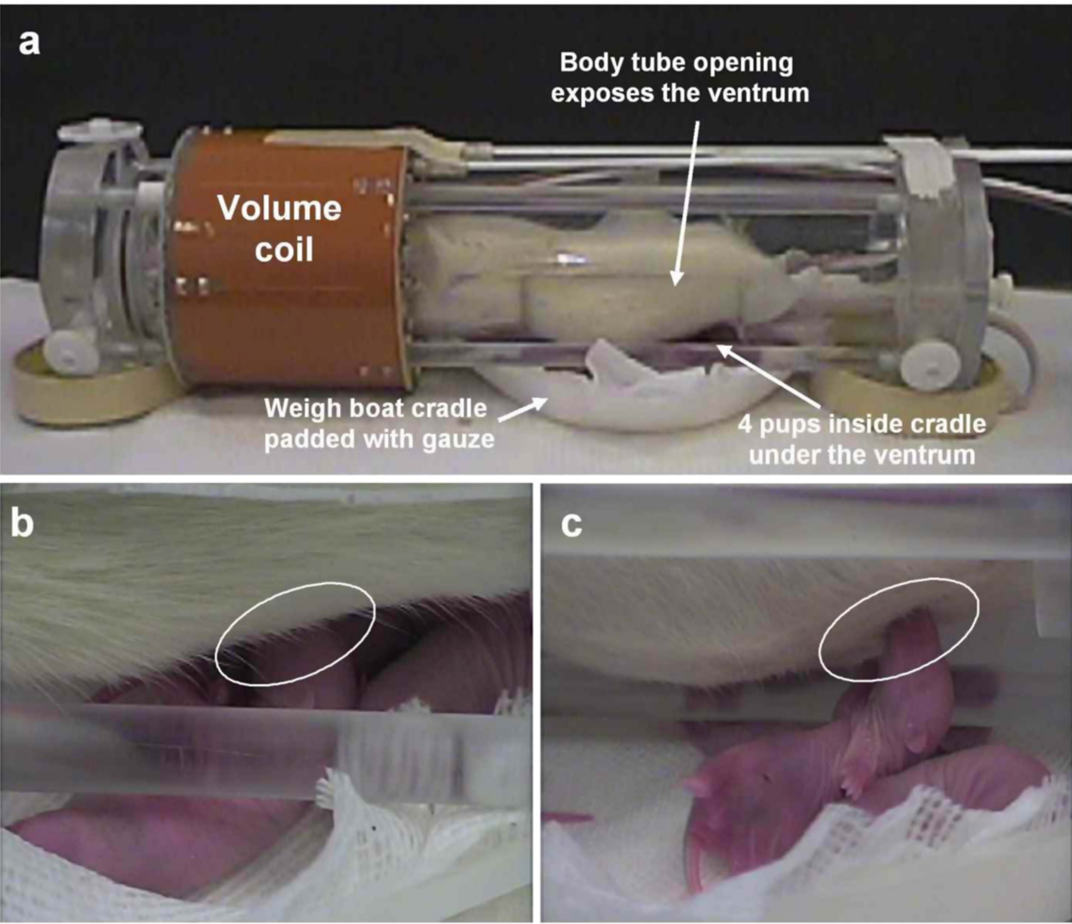


Fig. 7. Set-up for imaging awake lactating dams. Shown in (a) is a postpartum day 4–8 dam held in the body tune with an opening towards the ventrum that allows access to teats (shown in b and c). Adapted with permission from Febo et al. (2005b). (See Color Plate 25.7 in color plate section.)

weaning (Numan, 1994). Hormones combine with the physiology of parturition to foster maternal behaviour in first time mothers. However, from postpartum day 4 through postpartum day 20 the maintenance of maternal behaviour is more strongly influenced by learning and the tactile, auditory and odour stimuli coming from the pups.

Method for imaging the "nursing" brain

The methodology for imaging dams exposed to pup-suckling during a scanning session is published (Ferris et al., 2005). In brief, lactating dams are used between postnatal days 4–8. The adapted configuration used for maternal studies is shown in Fig. 7. For suckling studies, the hind limbs of the dams are loosely tethered and raised just above the floor of the body tube. This provided a visual inspection of the ventrum from outside the magnet and prevents the dam from kicking and injuring the pups during suckling. A cradle containing four to six pups is positioned under the dam in the magnet. The body tube has a window exposing the dam's ventrum to the pups. A thin plastic shield separates the pups from the mother. When the shield is pulled away the pups are exposed to the six hindlimb nipples and begin suckling. We are able to visually confirm when pups came onto the most caudal teats

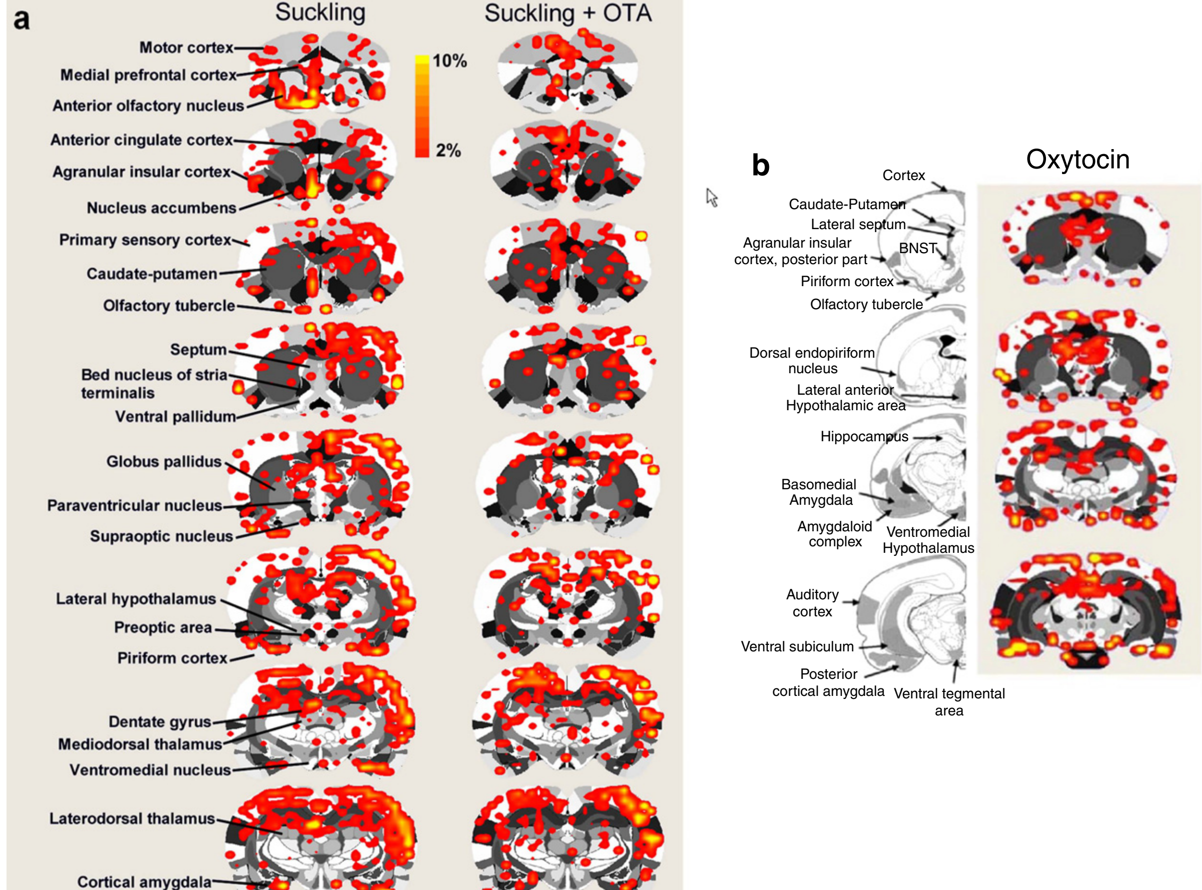


Fig. 8. BOLD activation maps for pup suckling with and without OXT and OXT antagonist. Composite brain maps of positive BOLD activity in response to suckling, suckling after oxytocin receptor blockade (a) and in response to oxytocin administration (b) in postpartum day 4–8 dams. Adapted with permission from Febo et al. (2005b). (See Color Plate 25.8 in color plate section.)

but are not able to determine whether all teats are suckled during the stimulation period. In all pup stimulation studies, suckling usually occurs within seconds of removing the shield. To promote suckling in the magnet, dams are prevented from having physical contact with their pups for 2 h prior to imaging. This is accomplished by inverting a shallow perforated Plexiglas box over the huddled pups.

Results

Pup suckling causes a robust activation of brain areas involved in olfactory, emotional and reward processing (Fig. 8). OXT administration alone without pup stimulation activates many of the same areas, for example the PVN, olfactory tubercle, anterior olfactory nucleus, insular cortex, piriform cortex, cortical amygdala, medial preoptic area and prefrontal cortex all show increases in BOLD signal to suckling and ICV oxytocin administration. When pup-suckling is preceded by OXT receptor antagonist there is suppression of activity in many of these areas.

Conclusion

These results provide evidence that OXT modulates brain activity during quiescent, or motorically inactive suckling. Several of these areas have been associated with the olfactory system, either indirectly or by direct projections received from the olfactory bulbs (Price, 1973). This suggests that OXT release during nursing contributes to olfactory-related neural activity. The activity in the PVN is not surprising since the PVN is involved in the milk-ejection reflex and OXT exerts a positive feedback effect on its own release from PVN neurons (Neumann et al., 1994). Importantly, the activated PVN is likely to be releasing OXT into central neural sites as well as into the periphery via the posterior pituitary. In the rat, destruction of the PVN delays the onset of maternal behaviours (Insel and Harbaugh, 1989). Similar effects have been observed by administering OXTA (van Leengoed et al., 1987). The present data provides evidence that OXT continues to be released within this area in fully maternal postpartum rats.

Final summary

These data from two unique experimental paradigms using fMRI, support and extend our previous understanding of AVP's and OXT's effect on brain activity under complex social and emotional conditions. Blocking V1a receptors suppresses aggressive motivation in bench-top studies and during imaging. The imaging data are characterized by a general reduction in BOLD signal particularly in areas identified as the putative neural circuit of aggressive motivation. However, this response is not due to a general decrease in arousal as sexually motivating stimuli activate primary olfactory and mesocorticolimbic dopaminergic systems in the presence of SRX251. Microinjection of AVP directly into the brain via the lateral cerebroventricle enhances BOLD signal in parts of the neural circuit of aggression that localize V1a receptors suggesting the decrease in aggression may be a direct effect of SRX251 on this neural circuit. Similarly, the neuranatomical substrates activated by OXT administration in the lactating rat closely parallel that observed with suckling stimulation alone. Moreover, blockade of OXT receptors with a specific antagonist selectively reduces brain activity in many of these common areas, evidence that endogenous OXT has a role in the neurobiology of nursing.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 26

Evolution of the arginine vasopressin 1a receptor and implications for mammalian social behaviour

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Abstract: Variation in social and mating behaviour among individuals or species can rarely be traced to its genetic basis. A notable exception is social behaviour in *Microtus* voles, which has been causally linked to polymorphisms in the *arginine vasopressin 1a receptor* (*Avpr1a*) gene region. A repetitive expansion in the promoter region of *Avpr1a* was interpreted as causally related to monogamy based on the results of inter-specific gene transfer, transcription assays and the presence–absence patterns in four *Microtus* species. The examination of further *Microtus* species and other mammals revealed that single or multiple short tandem repeats (STRs) upstream of *Avpr1a* are widespread in rodents. Phylogenetic analyses suggest that their absence in two closely related *Microtus* species is the result of an evolutionarily recent loss. Presence–absence patterns of the repetitive expansion show overall no association with mating and social system parameters in rodents. Similar STRs upstream of the *Avpr1a* gene in humans and primates are distinct in position and motif from those in rodents. Examination of the coding region (exon 1) of *Avpr1a* reveals unexpectedly high levels of genetic variation within the genus *Microtus* as well as in other mammalian taxa. Deleterious variation is largely eliminated by purifying selection on most regions of exon 1, but some sites in domains with particularly high rates of change are under positive selection in mammals. Variation in *Avpr1a* is likely of functional importance due to length variation in amino acid sequences, radical amino acid replacements and amino acid heterozygosity of individuals. It appears that intra-specific and intra-individual variation in both regulatory and coding regions deserves explicit consideration when causal links between genotype and phenotype are to be established.

Keywords: *Avpr1a*; *Microtus*; mating system; social behaviour; non-synonymous substitution; amino acid replacement; polymorphism; rodents

Introduction

It is a long-standing question whether human and animal behaviour has a heritable component and to what extent behavioural differences among individuals are caused by genetic polymorphisms. Recent advances in molecular biology, genetic engineering and large-scale DNA sequencing have provided ample evidence of behavioural traits under genetic control in diverse organisms. Many of the current examples result from a candidate gene approach since it has been realized that gene function is often conserved across species boundaries (Fitzpatrick et al., 2005). In this approach, genes that are known to influence behaviour in one organism are used as candidate loci for similar

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DOI: 10.1016/S0079-6123(08)00426-3

behaviour in other taxa. This transfer of hypotheses or knowledge across species boundaries often provides good starting points for the mechanistic investigation of the genetic bases of social behaviour of non-model organisms (Fitzpatrick et al., 2005). However, multi-species approaches and the integration of mechanistic and evolutionary analyses may also prove essential for a broader and more general understanding of the genetic basis of social behaviour as (Robinson et al., 2005). We describe in the following new insights resulting from bridging the gap between mechanistic and evolutionary approaches to mammalian social behaviour as exemplified by the *arginine vasopressin 1a receptor* (*Avpr1a*) gene. This shows in particular that even if the function and regulatory control of a gene have been resolved in detail for a particular system, consideration of the evolutionary background may provide important and unexpected implications for its generalizability.

Avpr1a: a single gene with major effect in mammals

Mammalian behaviour is often highly plastic and context-dependent, and its genetic bases are far from being understood. Like many other complex phenotypic traits, it is likely to be controlled by several genes, and their relative contributions and interactions are difficult to quantify (Bucan and Abel, 2002). A prime example for a single gene contributing a large effect to differences in mammalian social behaviour comes from detailed analyses of the *Avpr1a* gene (Keverne and Curley, 2004; Young and Wang, 2004; Fitzpatrick et al., 2005; Robinson et al., 2005). *Avpr1a* encodes the 1a receptor (V1aR) for the hormone arginine vasopressin (AVP). This system is involved not only in peripheral actions like water absorption and blood vessel control but also in cognitive functions in the brain such as memory and learning (Barberis et al., 1998). Polymorphism at the *Avpr1a* locus has been examined for association with human social behaviour and psychiatric disorders (Kim et al., 2002; Wassink et al., 2004; Bachner-Melman et al., 2005; Yirmiya et al., 2006), but the mechanics of how genetic variation translates into differences in social behaviour are much better known for *Microtus* rodents (Young et al., 1999; Hammock and Young, 2004; Lim et al., 2004a, b).

The structure of the Microtine *Avpr1a* and its flanking regions were detailed for two North American species of voles, *M. ochrogaster* and *M. montanus*, which differ in their social behaviour and in V1aR distributions in the brain (Insel and Young, 2001). The gene is relatively simply structured with two exons linked by an intron (Young et al., 1999). The region with presumable major impact on social behaviour, however, is the regulatory region a few hundred base pairs upstream of exon 1. The presence and absence of highly repetitive microsatellites or short tandem repeats (STRs) of several hundred nucleotides in the promoter region was found to be consistent with differences in partner preference and monogamy in four species of *Microtus* voles (Young et al., 1999). Experimental transfer of the coding region or the entire *Avpr1a* gene region including the STRs from monogamous *M. ochrogaster* to other rodents resulted in modified V1aR distributions in the brain and some behavioural changes in the target organisms. Transgenic male mice with the *Avpr1a* region from *M. ochrogaster* showed increased affiliative behaviour similar to *M. ochrogaster* (Young et al., 1999). Transgenic rats expressing additionally the *M. ochrogaster* V1aR showed improved social discrimination abilities and more active social behaviour (Landgraf et al., 2003). Socially promiscuous *M. pennsylvanicus* displayed increased partner preference formation when expressing V1aR from the socially monogamous *M. ochrogaster* additionally to the species-specific form (Lim et al., 2004b). Transcription assays demonstrated in vitro that length variation in the *Avpr1a*-STR in the promoter region may alter expression levels of a downstream gene (Hammock and Young, 2004). Longer repeat structures were, however, not generally associated with increased levels of gene expression but rather influenced expression in a cell-type specific manner. Breeding experiments with *M. ochrogaster* demonstrated predictable individual differences in receptor distribution patterns in the brain and in some social behaviours among males with STRs differing by less than 50 bp in length

(Hammock and Young, 2005). The observation of species differences in V1aR distribution patterns associated with differences in social behaviour of various mammals (Young, 1999), and the detection of repetitive structures upstream of the primate *Avpr1a* led to the hypothesis of a general connection between the evolution of social bonding and monogamy and the expansion of STRs in the promoter region (Lim et al., 2004a; Hammock and Young, 2005).

Avpr1a-STRs are not evolutionary switches

Recent analyses of a larger number of rodents and other mammals show that the evolution of monogamy in some *Microtus* species is generally independent from the expansion of the STR array upstream of the *Avpr1a* gene (Fink et al., 2006). The examination of the length and structure of the promoter region of *Avpr1a* in 21 *Microtus* species and other rodents showed that the long form of the *Avpr1a*-STRs is present in all but the two species *M. montanus* and *M. pennsylvanicus*. These two species are closely related and represent probably sister species (Fig. 1). Thus, several hundred nucleotides of the *Avpr1a*-STR sequence were probably lost in the common ancestor of *M. montanus* and *M. pennsylvanicus*, and the long form of *Avpr1a*-STRs is the ancestral state and not vice versa.

The evolutionary analysis of *Avpr1a*-STRs shows further that the presence of long *Avpr1a*-STRs in a species or population does not correlate with the social structure (Fink et al., 2006). Monogamy is generally rare among mammals, as less than 5% of the species show socially exclusive pair-bonding between a male and a female individual (Kleiman, 1977; Clutton-Brock, 1989). This low proportion is further diminished if the definition of monogamy is based on genetic analyses of reproductive patterns only, because such analyses detect frequently extra-pair reproduction of socially monogamous species (Reichard and Boesch, 2003). Detailed analyses of social or reproductive patterns are lacking for most of the 24 rodent species shown in Fig. 1. However, consistent with the expected rareness of monogamy, there is published evidence of social monogamy only for three species and of genetic monogamy for one of these species (Fig. 1). On the contrary, there is published evidence of social non-monogamy in eight species and of genetic non-monogamy in six species. More detailed genetic analysis of a further species (*M. arvalis*) demonstrated multiple paternity in litters (a conservative proxy for non-monogamy) in all investigated females even though these had all the long form of the *Avpr1a*-STRs. The observation of non-monogamy in many species with the long form of the *Avpr1a*-STRs shows clearly that the sole presence of these repeats is insufficient to explain the evolution of monogamy. Mechanisms triggered by these repeats may contribute to monogamous social behaviour somehow, but at least a second factor is required to explain differences between species. *Avpr1a*-STRs are therefore not simple evolutionary on/off switches for monogamy.

STRs are not homologous across mammals

It is further important to consider that repetitive structures upstream of *Avpr1a* are not conserved across mammal taxa. STRs are certainly homologous in regard to position and motif within the *Microtus* genus, but homology is difficult to establish with more distantly related rodents such as mouse and rat due to differences in repeat motifs (Fig. 2). The genomes of bonobo (*Pan paniscus*), chimpanzee (*Pan troglodytes*) or our own species show repetitive structures upstream of *Avpr1a* as well. These consist of very different repeat motifs compared to *Microtus*, and they are located much further upstream (~3.2 kb vs. ~0.6 kb in *Microtus*). Statistical associations of polymorphisms in these repeats have been reported for several forms of human social behaviour or psychiatric disorders (Kim et al., 2002; Wassink et al., 2004; Bachner-Melman et al., 2005; Yirmiya et al., 2006). It is currently unclear if these STRs are functionally linked to the expression of *Avpr1a* in primates (Young and Hammock, 2007). Contrary to coding regions, there is generally very little evidence for a functional role of STR polymorphism in non-coding regions for particular genes

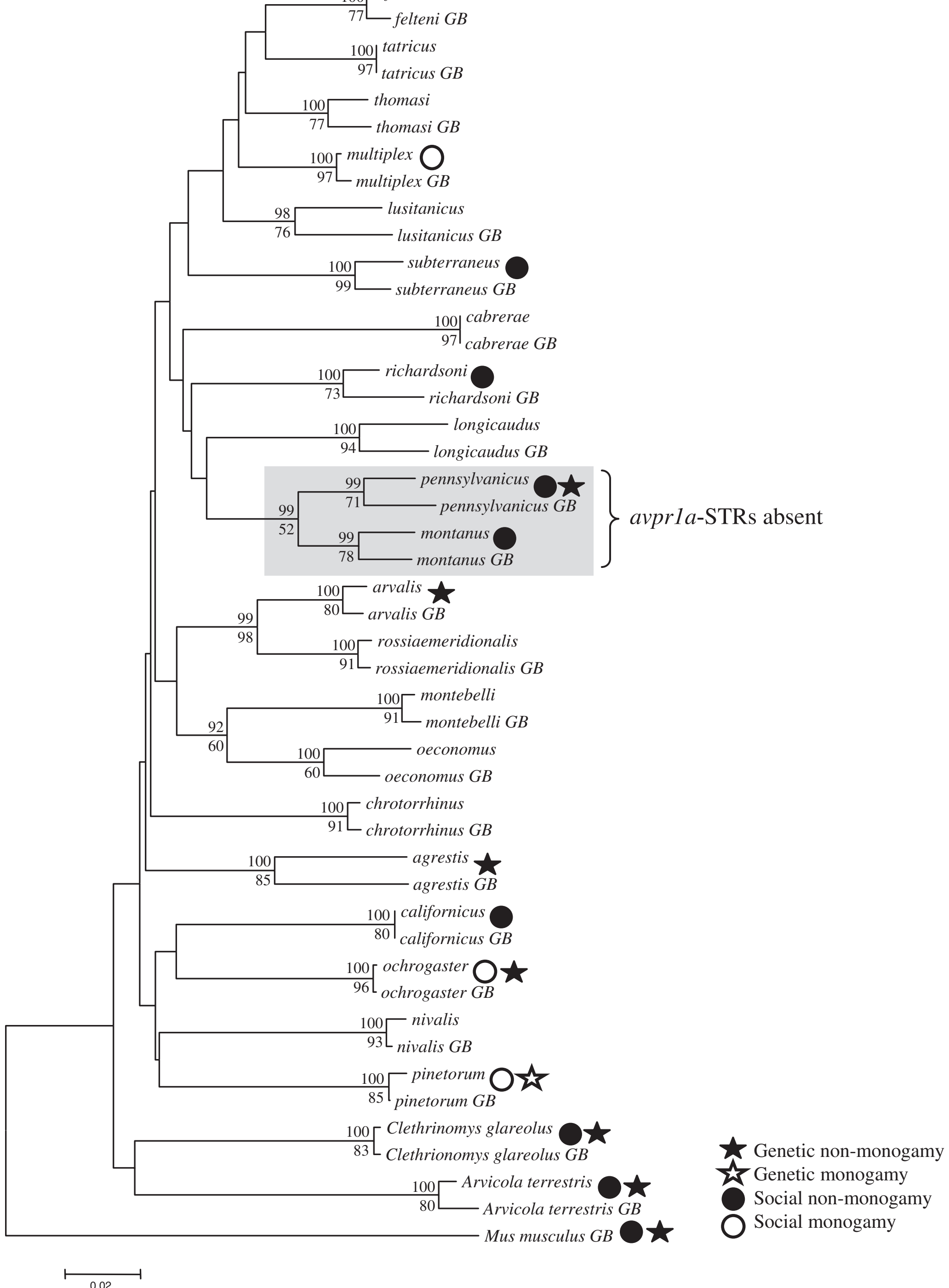

Fig. 1. Segregation pattern of *Avpr1a*-STRs and mating system parameters along a molecular phylogeny of *Microtus* and other rodents. The phylogeny was based on cytochrome *b* sequences of the analysed species in comparison with published molecular data from GenBank (GB). Bootstrap values >50 of neighbour-joining (above) and maximum likelihood (below) methods are shown on the branches. Symbols refer to genetic and social monogamy or non-monogamy. Long forms of *Avpr1a*-STRs are present in all species except for *M. pennsylvanicus* and *M. montanus* (highlighted in grey). There is no link between presence/absence of long *Avpr1a*-STRs and genetic or social monogamy. The deletion of the *Avpr1a*-STRs is monophyletic and represents an evolutionarily derived trait. Adapted with permission from Fink et al., 2006.

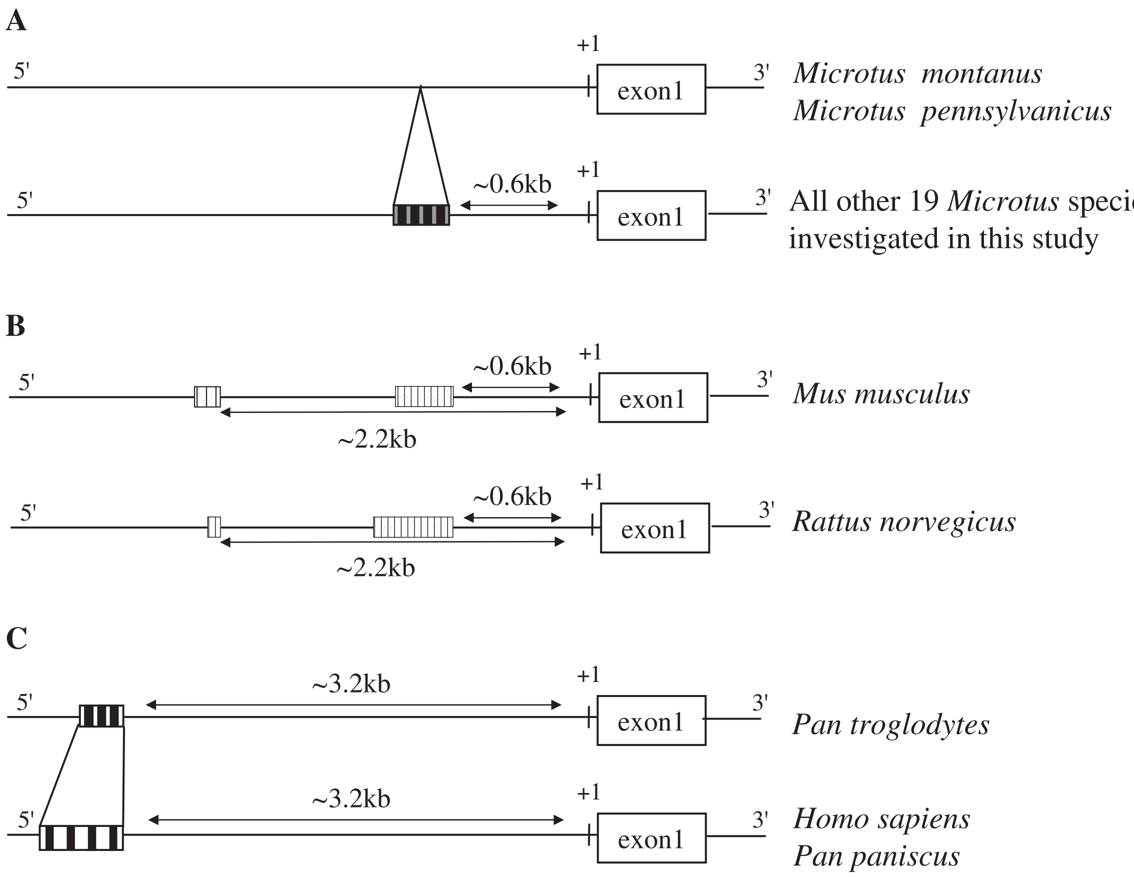

Fig. 2. Schematic view of STRs in the 5′ region of the *Avpr1a* gene of different mammals. (A) *Microtus* species exhibit a long stretch of STRs approximately 0.6 kb upstream of exon 1 of the *Avpr1a* gene except for two species, *M. montanus* and *M. pennsylvanicus*, which lack most of the repeats. (B) STRs are present in mouse (*Mus musculus)* and rat (*Rattus norvegicus*) at the same position as in *Microtus*, but they consist of different repeat motifs. An additional short array of STRs is located ~2.2 kb upstream of exon 1. (C) Humans (*Homo sapiens*), bonobo (*Pan paniscus*) and chimpanzee (*P. troglodytes*) show repetitive elements of different lengths approximately 3.2 kb upstream of exon 1. Repeat motifs of the primate STRs differ from those in rodents. Adapted with permission from Fink et al., 2006.

(Kashi and King, 2006). It is also important to note that STRs are very common elements in mammalian genomes. There are probably more than one million STR loci in the human genome, and these may constitute several percent of the total sequence length (Ellegren, 2004). It is therefore relatively likely to find an STR locus without functional relevance upstream of a given gene. However, even if this should be the case for the human *Avpr1a*, the physical linkage of STR alleles could make them valuable markers for functional variation in the gene itself (Young and Hammock, 2007).

Abundant variation in the coding part of *Avpr1a*

The extent of polymorphism in the coding parts of *Avpr1a* has received little attention compared to the promoter region. Mutations in the human AVP gene have been identified as causing, e.g. neurohypophyseal diabetes insipidus

(Rittig et al., 1996), and several single nucleotide polymorphisms have been found in *Avpr1a* (Kim et al., 2002; Saito et al., 2003; Wassink et al., 2004; Yirmiya et al., 2006) but it is unknown if these have consequences for the function of the encoded protein. Polymorphism in the coding part of the Microtine *Avpr1a* has been considered to be low and functionally negligible, based on the comparison of the DNA sequence of the two species *M. ochrogaster* and *M. montanus* (Young et al., 1999; Insel and Young, 2001; Nair and Young, 2006).

The comparison of the coding part of *Avpr1a* (exon 1) in a diverse range of mammals reveals extensive variation across all taxonomic levels down to polymorphism within individuals (Fink et al., 2007). Divergence in *Avpr1a* among higher mammal taxa is consistent with phylogenetic relationships as nucleotide or amino acid (AA) sequences of rodents or primates form clearly distinct groups (Fig. 3, Fink et al., 2007). The fact that human and vole *Avpr1a* are distinct is in agreement with other differences between the mechanisms of human bonding and vole social behaviour at the neurobiological and molecular level (Insel and Young, 2001; Young and Wang, 2004). AA changes in V1aR consist of substitutions as well as insertion–deletion polymorphisms resulting in length variation among taxa. Changes are more frequent in the ligand-binding domain

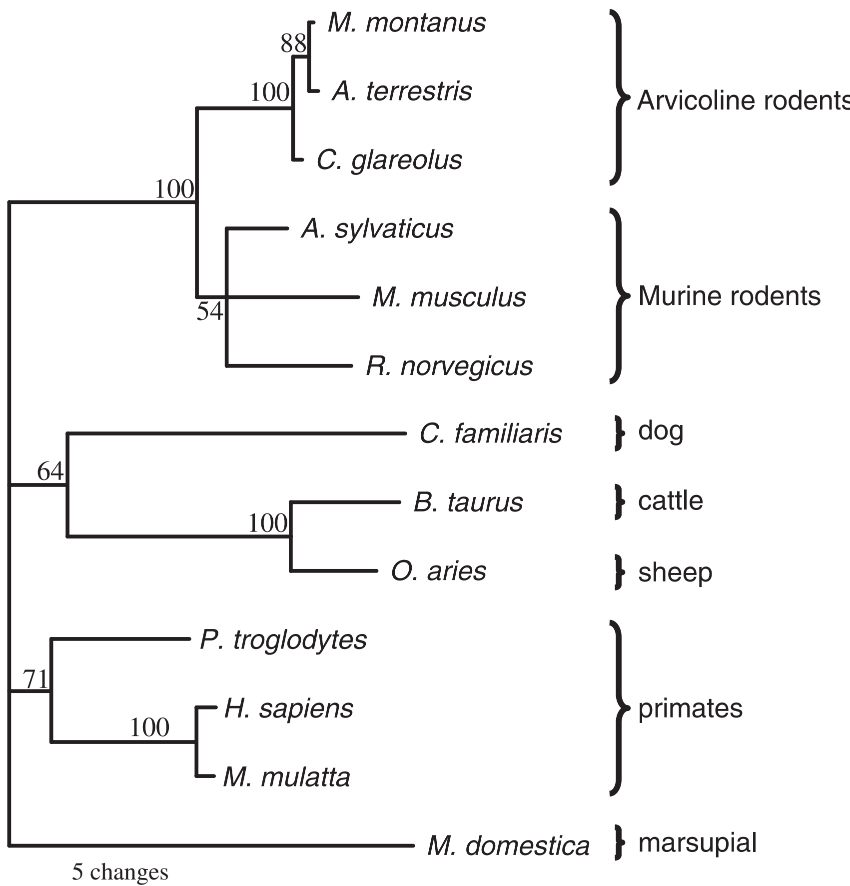

Fig. 3. Phylogenetic tree based on amino acid sequences of exon 1 of the *Avpr1a* gene of Eutherian mammals rooted with opossum (*Monodelphis domestica*). Bootstrap values >50 are shown on branches. The amino acid sequence is highly variable within mammals, and phylogenetic analyses reveal highly bootstrap supported clusters (e.g. rodents). After data from Fink et al., 2007.

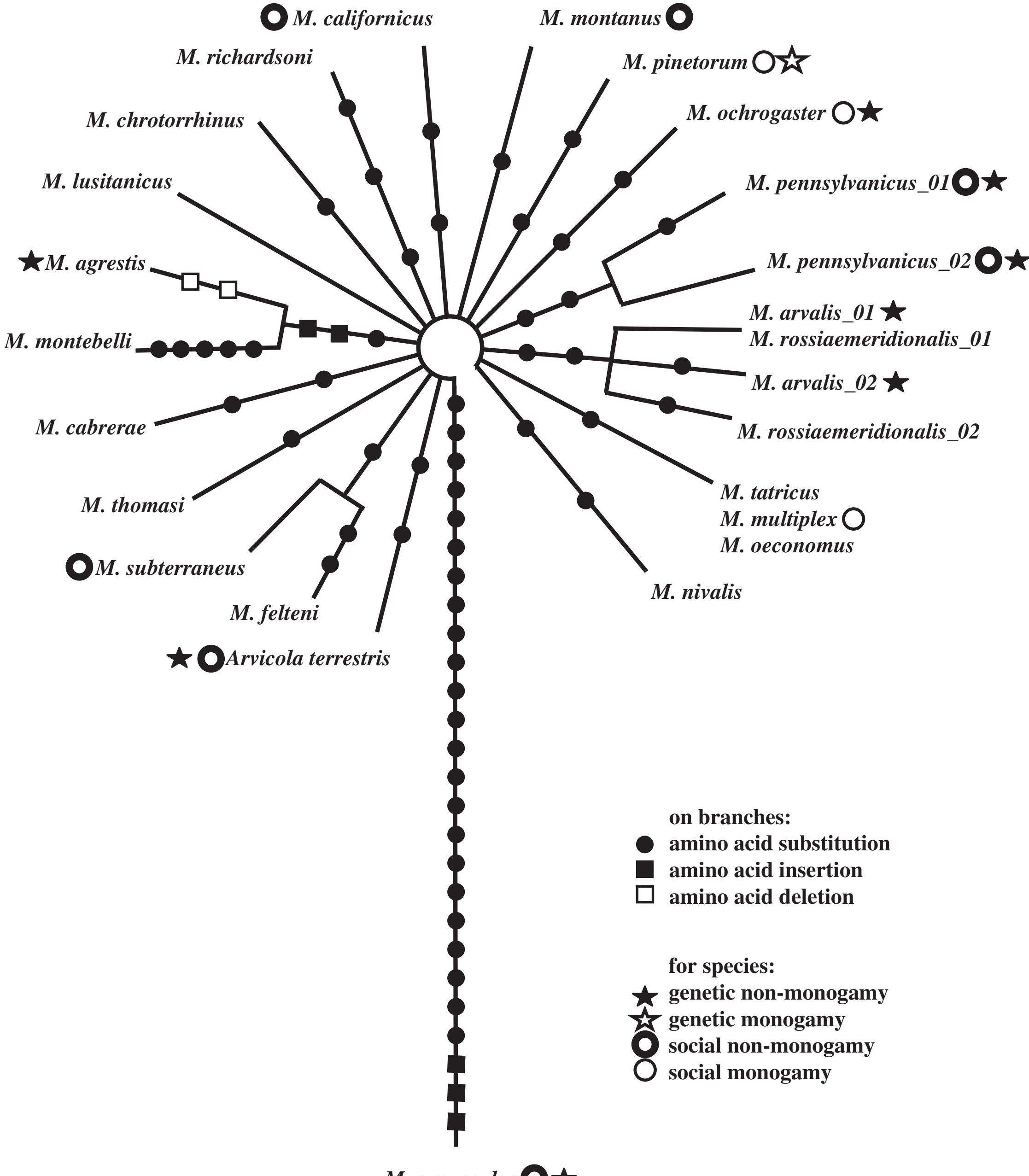

Fig. 4. Amino acid sequence tree of exon 1 of the *Avpr1a* gene of *Microtus* and other rodents. Symbols on branches represent amino acid substitutions, deletions and insertions, while symbols next to species names indicate genetic and social mating behaviour of species. Different amino acid sequences detected within the same individuals are labelled as 1 and 2. The majority of species displays unique sequence types but a few sequence types are shared between species (e.g. *M. tatricus, M. multiplex, M. oeconomus*). Consistent with general phylogenetic relationships in rodents, mice (*Mus musculus*) have a highly divergent amino acid sequence compared to voles, while the more closely related water vole (*Arvicola terrestris*) differs only by two amino acid substitutions (see also Fig. 3). There is no obvious link between the social or genetic mating system of a species and a certain amino acid substitution or protein type. After data from Fink et al., 2007.

and the G-protein-binding domain than in the trans-membrane parts of the receptor. Both regions show some evidence for positive selection, which may help to maintain variation despite purifying selection against deleterious variation on other parts of the receptor (Fink et al., 2007).

The evolutionary patterns observed among higher mammalian taxa extend also to the Microtine *Avpr1a*. Genetic diversity is much higher than previously assumed, and this variation seems at least in part relevant for the receptor function. Variation in the nucleotide sequence of exon 1 is substantial (13% variable sites among 24 *Microtus* species), and AA sequences show extensive polymorphism (Fig. 4, Fink et al., 2007). AA variation within the *Microtus* genus comprises insertion–deletion polymorphism as well as physico-chemically radical substitutions, and many of these changes are located in the ligand-binding domain. Consistent with phylogenetic expectations, Microtine and *Arvicola terrestris Avpr1a* are close to each other and very different from the house mouse (*Mus musculus*), but there is no obvious phylogenetic signal in AA variation within *Microtus* (Fig. 4). Some AA sequence types are shared between *Microtus* species (e.g. *M. tatricus*, *M. multiplex*, *M. oeconomus*), but individuals may even be heterozygous at the AA level (e.g. *M. pennsylvanicus*).

The large diversity of *Avpr1a* AA sequence types among closely related *Microtus* species shows clearly that this gene is not particularly conserved, and nucleotide substitution rates are indeed well within the range of other genes (Fink et al., 2007). Current data show no obvious link between particular AA sequences or AA substitutions and mating systems of the species (Fig. 4). The high diversity of AA sequence types could potentially mask such a relationship. It is important to consider, however, that V1aR is not only involved in the regulation of social behaviour but also in peripheral functions such as water retention (Barberis et al., 1998). The high level of variation may therefore, at least in part, be connected to the diversity of habitats and climates inhabited by the genus (Tamarin, 1985; Mitchell-Jones et al., 1999). Differences in environmental conditions and strong genetic structure within species provide the conditions for the evolution of potentially locally adapted V1aR types (Fink et al., 2004; Heckel et al., 2005; Schweizer et al., 2007). Thus, the analysis of phenotypic differences between species or populations (Cushing et al., 2001) will certainly benefit from taking potential protein variation within and among natural populations explicitly into account.

It appears further interesting to examine potential intra-individual variation of V1aR in more detail in the future. Earlier studies suggested that the expression of V1aR may occur in a tissue-specific manner (Hammock and Young, 2004). The detection of individuals that are heterozygous at the AA level poses the question if these express both protein variants, if expression is tissue-specific and if or to what extent hormone receptors in similar pathways may interact. The high level of *Avpr1a* AA sequence variability in *Microtus* and the amenability of these rodents to laboratory breeding provide suitable conditions to address these questions.

Conclusions

Mammalian social behaviour has a genetic component, and there is abundant evidence from the study of rodents that the AVP system contributes to it. It has become clear that large differences in mating systems and social behaviour between species and populations cannot be explained only by the presence or absence of a long array of STRs upstream of the *Avpr1a* gene. At the moment, we can neither discard polymorphisms in the regulatory region nor in the coding parts as contributing to the fine-tuning of social behaviour. It seems most promising to consider in future functional analyses of *Avpr1a*, explicitly the different levels of variation and their potential interactions with other hormone systems involved in similar regulatory circuits. This is a very challenging task, but new methodological developments and the combination of mechanistic and evolutionary approaches to the *Microtus* model and other mammals promise important insights for a broader understanding of the genetic basis of social behaviour.

Abbreviations

AA	amino acid
AVP	arginine vasopressin
Avpr1a	arginine vasopressin 1a receptor gene
STR	short tandem repeat or microsatellite
V1aR	arginine vasopressin 1a receptor

Acknowledgements

We thank Laurent Excoffier for his ongoing support, and all the persons and institutions providing sample material for our studies. This study was supported in part by grants from the Berne University Research Foundation and the Swiss National Science Foundation (Project no. 3100A0-112072).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 27

Oxytocin, vasopressin and sociality

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Abstract: The neurobiology of social behaviour is interwoven with autonomic, endocrine and other homoeostatic processes responsible for the adaptive functions of reproduction and survival. Young mammals are dependent on their mothers for nourishment, and the interaction between the mother and infant may be a physiological and neuroendocrine prototype for mammalian sociality. Although these adaptive functions of the mother–infant social behavioural dyad are obvious, adult social interactions, including social bonds, also are important to health and survival. Two neuropeptides, oxytocin (OXT) and arginine vasopressin (AVP), have been repeatedly implicated in mammalian social behaviours and emotional states that support sociality. Although best known for their roles in reproduction and homoeostasis, these peptides play a central role in the activation and expression of social behaviours and emotional states. Recent studies from our work with the prairie vole (*Microtus ochrogaster*), reviewed here, reveal a role for both OXT and AVP in behavioural and endocrine changes during social interactions, and also changes that are associated with the absence of social interactions (i.e. social isolation).

Keywords: oxytocin; vasopressin; prairie vole; social behaviour; autonomic nervous system; vagus; sex differences; isolation

Introduction

The prairie vole is a highly social rodent that is particularly dependent on social interactions and also neuropeptides for the regulation of endocrine and autonomic responses. Socially monogamous species, including prairie voles, share with humans a cluster of physiological and behavioural characteristics including the capacity to form social bonds and to develop extended families, which usually consist of a male and female pair and their offspring (Carter et al., 1995; Insel and Young, 2001). Socially monogamous rodents are especially sensitive to their social context, and have offered a particularly powerful model for understanding the mechanisms for positive social experiences, as well as mechanisms through which both negative and positive social experiences may have physiological consequences.

Neuroendocrine correlates of sociality

Sociality relies on sensory, autonomic, emotional and motor systems that permit or prevent approach or withdraw. Sensory and emotional

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DOI: 10.1016/S0079-6123(08)00427-5

processes are tuned to detect and interpret the features of social cues and to respond with synchronous autonomic reactions and appropriate motor patterns (Porges, 2007). In addition, selective social and emotional responses are implicit in the expression of social bonds (Carter and Keverne, 2002).

Mammalian neuropeptides, including OXT and AVP, integrate many processes including social behaviours, emotional feelings and responses, and activity in the autonomic nervous system. OXT facilitates mammalian birth, lactation and the development of maternal behaviours and social bonds. AVP plays a major role in fluid balance, and also in the regulation of cardiovascular and autonomic systems.

From an evolutionary perspective, OXT played a pivotal adaptive function in the survival of primates. As the central nervous systems and skulls of primates expanded, mechanisms evolved to facilitate birth and postnatal nourishment for the infant (Carter and Altemus, 1997). OXT facilitates the birth process through powerful muscle contractions. OXT may even protect the foetal nervous system during the birth (Tyzio et al., 2006). OXT also facilitates milk ejection and thus lactation. Lactation in turn permits the birth of immature infants, allowing postnatal cortical and intellectual development in young that are dependent on their mother as a source of both food and care-giving.

OXT sits at the center of a neuroendocrine network that coordinates social behaviours and concurrent response to various stressors, generally acting to reduce reactivity to stressors. OXT tends to decrease the withdrawal characteristics of fear and anxiety, and increase tolerance for stressful stimuli. OXT may protect the vulnerable mammalian nervous system from regressing into the primitive states of lower brainstem dominance (such as the ‘‘reptile-like’’ freezing pattern with an associated shut down of higher neural processes); mammals — with their comparative large cortices and a corresponding need for high levels of oxygen — can not endure long periods of hypoxia (Porges, 2007).

OXT is often released with and works in conjunction with AVP (Landgraf and Neumann, 2004). AVP is structurally similar to OXT, differing by only two of nine amino acids. The genes regulating the synthesis of these peptides are modifications of a common ancestral gene. The similarity of the OXT and AVP molecules also allows them to influence each other’s receptors. The actions of OXT and AVP are often — but not always — in opposite directions. AVP also plays a role in social behaviours and has adaptive functions in the face of behavioural and physiological stressors. However, the autonomic and emotional profiles of the effects of OXT and AVP are not identical. OXT tends to reduce behavioural and autonomic reactivity to stressful experiences. AVP, in contrast, is associated with arousal and vigilance, and in defensive behaviours, such territoriality and mate guarding (Carter, 1998; Landgraf and Neumann, 2004).

Various brainstem neural systems, including those that rely on peptides such as OXT and AVP, help to regulate emotional states including approach–avoidance reactions and the tendency of mammals to immobilize (Porges, 1998, 2001, 2007). OXT and AVP are synthesized in and are particularly abundant in the hypothalamus, but may reach distant receptors including those in the cortex and lower brain stem areas, such as the dorsal motor nucleus, responsible for autonomic functions. The consequence of these various actions of OXT and AVP are probably critical to the integration of behavioural, autonomic and emotional responses.

OXT and AVP have the capacity to move through the brain by diffusion, rather than acting only across a synapse or requiring transport by the circulatory system; for this reason these neuropeptides have pervasive effects on the central nervous system (Landgraf and Neumann, 2004). OXT in particular is unique in having only one known receptor and in using the same receptor for many functions, thus allowing coordinated effects on behaviour and physiology. There are three distinct receptor subtypes that bind AVP: V1a, V2 and V1b receptors. One, the V1a receptor, has been implicated in various kinds of social and defensive behaviours, and also helps to regulate blood pressure. Dynamic interactions between OXT and AVP may be of particular importance to the

approach and avoidance components of sociality (Carter, 1998). In humans, intranasal OXT facilitates "trust" behaviour, as measured in a computer game (Kosfeld et al., 2005), and the ability to detect subtle emotional cues from pictures of eyes (Domes et al., 2007). These studies support the hypothesis that OXT may have a central role in the behavioural responses necessary for social behaviour.

Consequences of social isolation

The importance of social interactions can be understood in part by examining the consequences of placing animals in social isolation (Table 1). Social isolation is a potent stressor, especially for highly social mammals such as prairie voles. In nature, female prairie voles rarely live alone, although males may sometimes be found in isolation.

When studied in the immediate postweaning period, both juvenile male and female prairie voles displayed isolation-induced increases in HPA axis hormones (CRF and corticosterone) (Ruscio et al., 2007a). In juvenile females, AVP-immunoreactive in the supraoptic nucleus (SON) also increased following isolation, but was lower in the paraventricular nucleus (PVN). In juveniles, isolation also was associated with a reduction in the birth of new cells (including neurons and glia) (Ruscio et al., 2005) and in males with an increase in the oestrogen receptor *alpha* (Ruscio et al., 2007b).

In adulthood, social isolation followed by an acute social stressor, was associated with increased OXT as well as AVP, CRF, ACTH and corticosterone (Grippo et al., 2007a, b). It is possible that the release of OXT under conditions of isolation/social stress may help to protect animals against the emotional effects of stress. This effect may be of particular importance in females, who may be especially vulnerable to the consequences of isolation (Grippo et al., 2007b).

Our recent research has demonstrated that at least 4 weeks of social isolation from a same-sex sibling induces behaviours relevant to both depression and anxiety using validated operational measures (Grippo et al., 2007a, b, f). For instance, compared to animals living in sibling pairs, after 4 weeks of isolation prairie voles showed increased immobility in a forced swim test, and reduced consumption of a palatable sucrose solution (Grippo et al., 2007b, f). In addition, isolated female prairie voles spent less time exploring the open arms of an elevated plus maze, suggesting an increase in anxiety (Grippo et al., 2007c, f).

Table 1. Effects in prairie voles of 3–4 weeks of social isolation from a sibling partner

Variables	Direction of change
Endocrine	
CRF-ir (CNS)	Increases (juveniles)
Corticosterone (blood)	Increase then returns to normal
ACTH	Increase
Vasopressin	
Supraoptic nucleus	Increase (juveniles)
Paraventricular nucleus	Decrease (juveniles)
Oxytocin (especially in females)	Increase (brain and blood)
Estrogen receptor alpha	Increase (juvenile males)
Social stressors	
Response to infant	
Attack behaviour	Increase
Response in resident intruder test	
Aggression	No difference
Reaction to intruder	Increase in heart rate (HR)
Recovery time	Increase time to HR recovery
Depression or anxiety related behaviours	
Sucrose intake	Decrease
Sucrose preference	Decrease
Forced swim test — immobility	Increase
Elevated plus maze — open arm	Decrease
Fecal boli	Increase
Autonomic measures	
Heart rate — basal	Increase
Heart rate — response to atropine	Increase
Heart rate — response to atenolol	Increase
HR response to social stressor	Increase
Vagal tone (RSA)	Decrease
Other	
Body weight	No difference
Heart weight	Increase
Neurogenesis	Decrease (esp. juveniles)

Using radiotelemetry, we have begun to investigate the autonomic responses in freely moving prairie voles, as a function of social housing with a sibling versus isolation. These studies indicate that prairie voles have a human-like autonomic nervous system, with high levels of vagal efferent activity, through the myelinated vagal pathways regulating the heart (Grippo et al., 2007d). For example, female prairie voles appear to exhibit a comparatively low resting heart rate and high HR variability, relative to other laboratory rodents such as rats and mice. Respiratory sinus arrhythmia (the modulation of heart rate synchronous with spontaneous breathing and mediated through myelinated vagal efferents) in the resting prairie vole has a peak-to-trough modulation of approximately 20–50 ms, which is in the range of healthy full-term human newborns (Porges, 1995). To investigate autonomic regulation of resting cardiac function in this species, autonomic blockade experiments were conducted using atenolol (8 mg/kg, ip) and atropine methyl nitrate (4 mg/kg, ip). Heart rate was slightly reduced under β-adrenergic receptor (sympathetic) blockade and increased under both cholinergic receptor (vagal) and combined autonomic blockade, suggesting a predominant vagal influence on resting heart rate in prairie voles. These findings are interesting given that adult prairie voles are very small (approximately 40–60 g), yet metabolically supported by a heart rate more like that of rats, which are typically 5–10 times larger. The sympathovagal balance in prairie voles, characterized by high vagal tone and low sympathetic tone at rest, is similar to humans and dogs, and differs from the more sympathetically mediated cardiovascular systems in other rodent species. In prairie voles isolation produces profound reductions in vagal control of the heart, as well as increases in sympathetic arousal and a reduced capacity to recover after a brief social stressor (a 5 min resident intruder test) (Grippo et al., 2007d). Thus, social isolation has potent inhibitory effects on various behavioural and autonomic measures, including reductions in sucrose preference (considered a measure of anhedonia), increased immobility in a forced swim test, and reduced exploration of a novel environment (used to index anxiety and fear), as well as increases in heart rate and inhibition of the vagal (parasympathetic) control of the heart.

More recently, we have found that OXT injections (given as daily subcutaneous injections for 2 weeks) were capable of preventing the effects of isolation on cardiac function and also prevented the loss of sucrose preference, which was seen in isolated animals receiving vehicle injections (Grippo et al., 2007e). These features of prairie voles physiology may be related to the high levels of sociality shown in this species and also may be a model for understanding the role of the autonomic nervous system, and especially the more recently evolved, myelinated vagal pathways, in social behaviour.

OXT and AVP receptors are found in many limbic structures including the extended amygdala. The amygdala and its connections serve a role in the integration of reactions to various kinds of sensory stimuli, including approach and avoidance (Davis, 2006). In human males, intranasal administration of OXT inhibited the activity of the amygdala in response to social stimuli and altered down-stream connections to brainstem structures involved in the regulation of the autonomic nervous system (Kirsch et al., 2005). AVP, acting centrally (in areas including the bed nucleus of the stria terminalis (BNST), amygdala and lateral septum), may elevate vigilance and defensiveness, possibly serving in some cases as an antagonist to the effects of OXT — and vice versa (Huber et al., 2005). Behaviours mediated by the central amygdala may mediate stimulus-specific fear, while the BNST has been implicated in experiences related to anxiety. Other peptides, including CRF, released during "stressful" experiences may be anxiogenic, acting in the extended amygdala including the BNST, to influences responses to dangerous or threatening cues (Davis, 2006). At least some of the fear-associated or defensive actions of CRF or AVP can be counteracted by OXT. Thus, OXT may have the capacity to reduce fear and calm the sympathetic responses to stressful stimuli, including those associated with social behaviour.

Sex differences in sociality

Explanations for sex differences in sociality have typically focused on steroid hormones. However, neuropeptides also may be involved. For example, the hypothalamic synthesis of AVP is androgen-dependent and AVP may be of particular importance to social behaviour and anxiety behaviour in males (De Vries, 2004; Bielsky et al., 2005; Carter, 2007). OXT is oestrogen-dependent, but has functions in both males and females. Working together these molecules may allow sexually dimorphic responses to emotionally contradictory tasks such as being social to a mate or forming social bonds, while also permitting rapid behavioural and autonomic reactions, including defensive behaviours or aggression, in the face of other social cues. In addition, OXT receptors have been found in midbrain regions which organize defensive motor behaviours and autonomic states and are assumed to down regulate these circuits under contexts of safety.

Elevations in OXT during periods of isolation in prairie voles are also sexually dimorphic, with females more likely than males to show increases in OXT (Grippo et al., 2007b). In human females increases in OXT were associated with "gaps in social relationship" (Taylor et al., 2006). The significance of isolation-related elevations in OXT remains to be empirically determined, but it is likely that OXT is a component of a homoeostatic process that helps mammals deal with isolation or other stressful experiences. Such findings also might facilitate preparedness for social engagement and social contact, especially adaptive in females who may be less able than males to cope with isolation.

Because AVP is sexually dimorphic in the extended amygdala and lateral septum (higher in males) it is also a candidate for a role in explaining sex differences in sociality. For example, males versus females may experience or respond to social stimuli using sexually dimorphic neural pathways to accomplish similar goals (De Vries, 2004). AVP has been implicated in situations in which an active strategy is required for an effective response, while OXT might be associated with a more passive coping strategy (Koolhaas et al., 1998). There is evidence in humans that centrally administered AVP has different behavioural effects in males versus females (Thompson et al., 2006). In addition, mutation of the AVP V1a receptor was more disruptive in males than females (Bielsky et al., 2005).

Individual or sex differences in the genetics of this system may be associated with individual differences in sociality. It is likely that OXT and AVP play central roles in mental health. In addition, disruption in these systems may be associated with mental illnesses in which disruptions in social and emotional behaviours are features of the disorder. For example, the genetic substrates responsible for the production of OXT and AVP receptors have been linked to disorders such as autism (reviewed Jacob et al., 2007; Carter, 2007).

Acknowledgements

This research was supported by grants from the National Institutes of Health: MH73233 (Angela J. Grippo), MH67446 (Stephen W. Porges), and MH72935 and HD38490 (C. Sue Carter) and the Institute for Research on Unlimited Love.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 28

Neuropeptides and social behaviour: effects of oxytocin and vasopressin in humans

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Abstract: The fundamental ability to form attachment is indispensable for human social relationships. Impairments in social behaviour are associated with decreased quality of life and psychopathological states. In non-human mammals, the neuropeptides oxytocin (OXT) and arginine vasopressin (AVP) are key mediators of complex social behaviours, including attachment, social recognition and aggression. In particular, OXT reduces behavioural and neuroendocrine responses to social stress and seems both to enable animals to overcome their natural avoidance of proximity and to inhibit defensive behaviour, thereby facilitating approach behaviour. AVP has primarily been implicated in male-typical social behaviours, including aggression and pair-bond formation, and mediates anxiogenic effects. Initial studies in humans suggest behavioural, neural, and endocrine effects of both neuropeptides, similar to those found in animal studies. This review focuses on advances made to date in the effort to understand the role of OXT and AVP in human social behaviour. First, the literature on OXT and AVP and their involvement in social stress and anxiety, social cognition, social approach, and aggression is reviewed. Second, we discuss clinical implications for mental disorders that are associated with social deficits (e.g. autism spectrum disorder, borderline personality disorder). Finally, a model of the interactions of anxiety and stress, social approach behaviour, and the oxytocinergic system is presented, which integrates the novel approach of a psychobiological therapy in psychopathological states.

Keywords: neuropeptides; oxytocin; arginine vasopressin; social behaviour; stress; anxiety; attachment; approach behaviour

Introduction

Social interaction permeates the whole of human society and the fundamental ability to form attachment is indispensable for human social relationships. Impairments in social behaviour are associated with decreased quality of life and pathological states. In view of the ubiquity of abnormal social behaviour in mental disorders, Insel (2002) noted, “We are, by nature, a highly affiliative species craving social contact. When social experience becomes a source of anxiety rather than a source of comfort, we have lost something fundamental — whatever we call it” (p. 3). In non-human mammals, receptors for the neuropeptides oxytocin (OXT) and arginine vasopressin (AVP) are distributed in various brain regions (Landgraf and Neumann, 2004) associated with the central nervous control of stress and anxiety and with social behaviour, including parental care, pair-bonding, social memory, and social aggression. Specifically, OXT seems both to enable animals to

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DOI: 10.1016/S0079-6123(08)00428-7

overcome their natural avoidance of proximity and to inhibit defensive behaviour, thereby facilitating approach behaviour (Carter and Altemus, 1997; Pedersen, 1997; Carter, 1998; Uvnas-Moberg, 1998; Insel and Young, 2001; Young et al., 2002). AVP has primarily been implicated in male-typical social behaviours, including aggression, pair-bond formation, scent marking, and courtship (Carter, 1998; Young and Wang, 2004; Lim and Young, 2006).

Aside from its effects on social behaviour, OXT shows significant binding in the limbic system, including the amygdala (Landgraf and Neumann, 2004; Huber et al., 2005) and decreases anxiety and the neuroendocrine response to stress in social interactions (Windle et al., 1997a, 2004; Neumann et al., 2000a; Bale et al., 2001; Carter et al., 2001; Parker et al., 2005). In contrast, AVP seems to play an anxiogenic role, with elevated AVP expression in the hypothalamic paraventricular nucleus being associated with increased behavioural and neuroendocrine anxiety levels (Murgatroyd et al., 2004). In addition, Ferris et al. (2006) recently showed that the orally active AVP V1a receptor antagonist SRX251 selectively blocks aggressive behaviour in hamsters. At a cellular level, Huber et al. (2005) recently showed that distinct populations of neurons in the amygdala are activated by OXT and AVP receptor stimulation, through which these peptides modulate the integration of excitatory information from the amygdala and cerebral cortex in opposite manners. These results suggest that the endogenous balance between OXT and AVP receptor expression and activation may set distinct, individually tuned levels for the activation of the autonomic fear response. In general, centrally active AVP seems to be associated with increased vigilance, anxiety, arousal, and activation, while OXT has behavioural and neural effects associated with reduced anxiety, relaxation, growth, and restoration (Carter, 2007). Thus, both peptide hormones are important in social stress and in social interaction, and in turn, a dysregulated metabolism may be associated with mental disorders of psychosocial relevance.

Much of the knowledge regarding the ability of OXT and AVP to regulate social interactions is based on data from animals using centrally administered agonists and antagonists or knockout mice. However, initial studies suggest similar social and stress-related effects of both neuropeptides in humans (for review, see Bartz and Hollander, 2006; Heinrichs and Gaab, 2007). Besides the endogenous stimulation of OXT during breast-feeding and positive physical contact, leading to attenuated endocrine responses to stress in women (Altemus et al., 1995; Turner et al., 1999; Heinrichs, 2000; Light et al., 2000; Heinrichs et al., 2001, 2002; Ditzen et al., 2007), studies in humans have also been carried out with exogenous administration of OXT and AVP. Although intravenous OXT infusion has been shown to induce significant behavioural effects (Hollander et al., 2003, 2007), it seems that only a small part of the neuropeptide passes the blood–brain barrier, and possible side effects are more likely following intravenous infusion of neuropeptides. In particular, a potential clinical use is dependent on a more direct and secure pathway to the human brain. Fortunately, neuropharmacological research has shown that neuropeptides gain access to the human brain after intranasal administration (Pietrowsky et al., 1996; Born et al., 1998, 2002; Heinrichs, 2000), providing a useful method for studying the central nervous effects of OXT and AVP in humans (Heinrichs and Gaab, 2007).

This article reviews recent advances made to date in the endeavour to understand the role of OXT and AVP in human social behaviour. As the animal literature in this area is reviewed in detail by several other authors in this issue, we will focus on the existing findings from studies of healthy humans and patients. In the first part of this review, we examine the significance of OXT in stress responsiveness, anxiety, and prosocial behaviour. In the second part, we address the role of AVP in social behaviour. Finally, we conclude by outlining the clinical implications for mental disorders that are associated with social deficits and present a model of the interactions of anxiety and stress, social approach behaviour, and the oxytocinergic system.

Effects of oxytocin on human social behaviour

Oxytocin, social stress and anxiety

In animal studies, OXT has been found to be released peripherally and within the brain in response to

both physical and psychological stress and fearful situations (Neumann et al., 2000a, b). Intracerebral OXT has been shown to inhibit the stress-induced activity of the hypothalamic-pituitary-adrenal (HPA) axis responsiveness (Neumann, 2002; Parker et al., 2005) and the activity of the amygdala in the modulation of the autonomic fear response (Huber et al., 2005). Numerous studies on the inhibitory influence of OXT on stress-responsive neurohormonal systems focused on the endogenous stimulation of OXT during lactation in rodents. The suckling stimulus by the newborn was found to increase OXT release and decrease basal plasma levels of ACTH and cortisol (Uvnas-Moberg, 1994; Carter and Altemus, 1997; Windle et al., 1997b; Uvnas-Moberg et al., 1999; Neumann et al., 2000b; Carter et al., 2001).

In lactating women, the increase of OXT following breast-feeding is associated with dampened levels of ACTH and cortisol (Chiodera et al., 1991; Amico et al., 1994; Nissen et al., 1996; Heinrichs et al., 2002). In addition, lactation in humans also appears to reduce responses to physical and psychosocial stress exposure. In lactating women, attenuated HPA axis responses can be observed if breast-feeding starts 30–60 min before stress exposure, depending on the kind of stressor (Altemus et al., 1995, 2001; Heinrichs et al., 2001). As no effect of stress has been found on OXT plasma levels, OXT does not seem to mediate the attenuation of cortisol stress responses at the adrenal level (Heinrichs et al., 2002). Thus, the inhibitory effect of OXT on HPA axis responsiveness points to a more central modulation and could, in fact, be localized in the paraventricular nucleus and in the septum, as demonstrated in rats (Neumann et al., 2000a, b). Interestingly, breast-feeding mothers with increased plasma OXT in response to a speech stressor that immediately followed baby-holding were found to have lower blood pressure than mothers with a decrease in OXT after stress (Light et al., 2000). Furthermore, non-postpartum healthy women who showed increased plasma OXT levels in response to positive emotion and massage and who maintained OXT levels during negative emotion were less likely to report interpersonal problems associated with intrusiveness (Turner et al., 1999). Maintaining OXT levels during sadness has also been associated with lower anxiety in close relationships. Recently, Ditzen et al. (2007) showed that women receiving standardized physical contact from their partner (neck and shoulder massage) before stress exposure exhibited significantly lower cortisol and heart rate responses to stress compared with women who received verbal social support or no social interaction from the partner. Altogether, these results from human studies imply a direct protective effect of endogenous OXT stimulation.

Within this context, however, it should be noted that there are a variety of confounding factors, in particular the release of other hormones (e.g. prolactin or opioid peptides), which are difficult to control for in endogenous stimulation paradigms such as lactation or physical contact. Moreover, plasma concentrations of OXT do not seem to reflect the central nervous availability of the neuropeptide (Landgraf and Neumann, 2004). Thus, the specific effects of central OXT as an underlying biological mechanism for the reduction of stress and anxiety in humans have to be investigated using challenge procedure methodologies involving OXT administration in double-blind, placebo-controlled designs.

In an initial study, we were interested in investigating the interactive effects of an altered availability of central nervous OXT and social support in a standardized psychosocial stress protocol (Heinrichs et al., 2003). In a double-blind, placebo-controlled design, all participants were randomly assigned to receive intranasal OXT (24 IU) or placebo 50 min before stress, and either social support from their best friend during the preparation period or no social support. Subjects who received both social support and intranasal OXT exhibited the lowest cortisol concentrations during stress exposure, whereas subjects who received no social support and placebo demonstrated the highest cortisol response (Heinrichs et al., 2003). Notably, there were corresponding results in psychological measures, indicating that subjects without social support and with placebo showed the expected decrease in calmness and increase in anxiety during stress. In contrast, participants who received either social support or OXT or both protective factors showed increasing calmness and decreasing anxiety scores during stress. Moreover, pre- and post-stress

comparisons of anxiety showed an anxiolytic effect of OXT administration. From these data, it may be concluded that OXT plays an important role as an underlying biological mechanism for the well-known stress-protective effects of positive social interaction.

Recent animal research indicates that central nervous OXT modulates the autonomic fear response via OXT receptors in the amygdala (Huber et al., 2005). In an initial functional magnetic resonance imaging (fMRI) study in humans, Kirsch et al. (2005) imaged amygdala activation through fear-inducing visual stimuli in healthy men after double-blind, placebo-controlled crossover substance administration. The authors found that 27 IU intranasal OXT markedly reduced activation of the amygdala and reduced coupling of the amygdala to brainstem regions implicated in autonomic and behavioural manifestations of fear. Most recently, we extended these results by measuring neural responses to social cues with different emotional valences: emotional faces with fearful, angry and happy expressions (Domes et al., 2007a). In an fMRI study using a double-blind, placebo-controlled within-subject design, we found that a single dose of 24 IU OXT reduced right-sided amygdala responses to all three face categories even when the emotional content of the presented face was not evaluated explicitly. In addition, exploratory whole brain analysis revealed modulatory effects in prefrontal and temporal areas, as well as in the brainstem (Domes et al., 2007a). In conclusion, these initial neuroimaging studies suggest a modulatory role of OXT on amygdala responsiveness irrespective of the emotional valence of stimuli. The attenuating effect on amygdala activity in response to both positive and negative social stimuli might reflect reduced uncertainty about the predictive value of a social stimulus and thereby facilitate social approach behaviour.

Oxytocin, social cognition and social approach

Besides its modulating role in psychosocial stress, OXT is involved in the regulation of social approach behaviour, social affiliation, and attachment. A large body of evidence from animal studies has implicated OXT and AVP in mating, pair-bonding, and adult–infant attachment (Lim and Young, 2006). It is well known that pair-bonding in prairie voles, for example, is regulated by both OXT and AVP (Cho et al., 1999), whereas maternal behaviour in rats is modulated only by OXT (Insel, 1992). In addition, aggressive behaviour seems to be modulated selectively by AVP (Ferris et al., 1992).

In contrast to the long tradition of animal research, human studies have only just begun to gain insights into how OXT modulates social approach behaviour and affiliation including the associated cognitive processes. In a first study, OXT was found to increase the stress-reducing and anxiolytic effect of social support in a psychosocial laboratory stress protocol. Participants who had received OXT in combination with social support from their best friend showed significantly attenuated endocrine and behavioural stress responses compared with social support alone (Heinrichs et al., 2003). In another study on the effects of OXT on human memory, OXT selectively modulated implicit memory depending on the social relevance (reproduction-related vs. neutral) of semantic word stimuli (Heinrichs et al., 2004).

In humans, trust in other people is a prerequisite of social affiliation and social approach. Therefore, the experiment by Kosfeld et al. (2005) can be seen as a pivotal study addressing the role of OXT in human social approach behaviour. The authors showed that a single dose of 24 IU intranasal OXT caused a substantial increase in trust among humans, thereby greatly increasing the benefits from social interactions in a trust game. More specifically, 45% of subjects in the OXT group showed the maximal trust level, whereas only 21% in the placebo group showed maximal trust. Most importantly, this study shows that the effect of OXT on trust was not due to a general increase in the readiness to bear risks. Rather, OXT specifically increases an individual's willingness to accept social risks within social interactions. These results concur with animal research suggesting an essential role for OXT as a biological basis of prosocial approach behaviour in humans.

Focusing on the modulation of social cognitive processes, a recent study from our laboratory examined the effects of intranasally administered OXT on the ability to infer the affective state of another individual from facial cues (Domes et al.,

2007b). In this study, participants were given a set of pictures showing the eye region of emotional faces, and were asked to infer the internal state of the depicted person — a test originally developed for the assessment of social attributional deficits of "mind-reading" in autism spectrum disorder (ASD) (Baron-Cohen et al., 2001; Dziobek et al., 2006). A single dose of 24 IU OXT administered intranasally enhanced performance in this test compared to placebo. Thus, OXT improves the ability to infer the mental state of others. Although the causal mechanisms are not clear, OXT-induced facilitation of certain social cognitive functions might be associated with social approach behaviour. A recent study by Guastella et al. (2008) reported an increased number and duration of gazes towards the eye region of emotionally neutral human faces following intranasal OXT administration (24 IU) as compared to placebo (Guastella et al., 2008), indicating a key role of OXT in facial processing and interpersonal communication in humans.

Taken together, the recent studies suggest that in humans too, OXT modulates social perception, social cognition, and social behaviour, thereby possibly promoting social approach and affiliation. Besides the stress-reducing and anxiolytic effects, OXT seems to be involved in social cognitive functions such as emotion recognition. Functional imaging studies support the idea that the anxiolytic effect of exogenously administered OXT is at least in part due to a deactivation of amygdala-mediated arousal. Reduced emotional arousal during social encounters might also promote social approach and might therefore contribute to the positive effects of OXT on trust and social cognition. Clearly, alternative pathways will need to be investigated in future research, given the widespread distribution of OXT receptors in the brain (Landgraf and Neumann, 2004) and the distribution of the neural network underlying social cognition and emotion (Adolphs, 2003).

Effects of arginine vasopressin on human social behaviour

Whereas OXT plays a key role both in prosocial behaviour and in the central nervous control of stress and anxiety, AVP has primarily been implicated in male-typical social behaviours, including aggression and pair-bond formation and in stress responsiveness (Goodson and Bass, 2001). Although most of the studies conducted thus far on human social behaviour have focused on OXT, few studies on AVP suggest behavioural effects similar to those found in animal research.

To examine the facilitatory role of central AVP in human aggressive behaviour, Coccaro et al. (1998) examined the relationship between cerebrospinal fluid (CSF) AVP and indices of aggression in personality-disordered subjects. The authors found a positive correlation between levels of CSF AVP and life histories of general aggression and aggression against other persons, suggesting an enhancing effect of central AVP in individuals with impulsive aggressive behaviour.

Using a laboratory challenge methodology, two recent studies examined the effect of intranasal AVP administration on human facial responses related to social communication. In a first study, Thompson et al. (2004) examined the effects of 20 IU intranasal AVP on cognitive, autonomic, and somatic responses to emotionally expressive facial stimuli in healthy male students using a placebo-controlled, double-blind design. Whereas AVP did not affect attention towards, nor autonomic arousal in response to, emotional facial expressions with different valence (neutral, happy, angry), the authors observed selective enhancements of the corrugator supercilii electromyogram (EMG) responses evoked by emotionally neutral facial expressions. Interestingly, subjects of the AVP group yielded magnitudes in response to neutral facial expressions that were similar to placebo subjects' magnitudes in response to angry facial expressions (Thompson et al., 2004). Due to the crucial role of this muscle group for species-specific agonistic social communication (Jancke, 1996), these results suggest that AVP may influence aggression by biasing individuals to respond to emotionally ambiguous social stimuli as if they were threatening or aggressive.

In order to investigate possible sex-specific influences of AVP on human social communication, Thompson et al. (2006) conducted a further experiment. Men and women received 20 IU

intranasal AVP or placebo, and their facial EMG, heart rate, and skin conductance responses to pictures of same-sex models posing various facial expressions of emotion were tested. In addition, subjects rated the friendliness of the faces. In men, AVP stimulated agonistic facial motor patterns in response to the faces of unfamiliar men. Interestingly, AVP also decreased perceptions of the friendliness of these faces. In women, by contrast, AVP stimulated affiliative facial motor patterns in response to unfamiliar female faces and increased perceptions of friendliness of these faces. Notably, AVP also affected autonomic responses to threatening faces and increased anxiety.

Altogether, central AVP has the ability to influence social communication processes in humans, as is the case in numerous other vertebrates. Moreover, the effects of AVP appear to be sex-specific, promoting agonistic and affiliative types of responses towards same-sex faces in men and women, respectively.

Clinical implications

Social behaviour in health is tightly regulated and dysfunctional alterations can result in a psychopathological state. Aside from social anxiety, social deficits may also occur as ASDs, obsessive-compulsive disorder (OCD) or as borderline personality disorder (BPD). In the following, we discuss the role of OXT and AVP in mental disorders that are associated with social deficits.

Autism spectrum disorder

ASD is a group of developmental disorders comprising autism, Asperger syndrome, and high functioning autism (DSM-IV). ASD is characterized by a specific pattern of abnormalities in communication, impairments in social cognition and repetitive behaviours. It has been argued that deficits in theory of mind or "mind-reading", that is the ability to attribute mental states to behavioural cues, represent the core deficit underlying the social impairments of ASD (Frith and Happe, 1994; Frith, 2001; Schultz, 2005).

Because some social deficits in ASD mimic the deficits of animals that lack OXT, some authors have argued that there might be a link between ASDs and OXT/AVP (Insel, 1997; Young et al., 2002; Hammock and Young, 2006; Carter, 2007). We will review three lines of evidence that support this notion: (1) plasma-level studies, (2) genetic studies, and (3) administration studies in ASD.

First, there is some evidence that patients with ASD show blunted plasma levels of OXT. Modahl et al. (1998) found lower plasma levels in children with ASD and correlations between plasma OXT levels and social functioning. Green et al. (2001) extended these results by demonstrating alterations in OXT metabolism in ASD. In particular, children with ASD showed enhanced OXT precursor to OXT ratios.

The second line of evidence highlights the possible role of the OXT receptor (*Oxtr*) gene in ASD. A number of studies have emphasized the 3p25 region containing the *Oxtr* gene as the most promising linkage site for ASD (McCauley et al., 2005; Lauritsen et al., 2006; Ylisaukko-oja et al., 2006). A study with a sample of Chinese Han families suggests an association between ASD and two single nucleotide polymorphisms (rs2254298 and rs53576) (Wu et al., 2005). These results were confirmed in part in a Caucasian sample (Jacob et al., 2007) and further extended in a family-based association study (Lerer et al., 2007) showing interactions with social cognitive skills. Despite these studies, which revealed associations of certain *Oxtr* polymorphisms with ASD, there are other studies suggesting that polymorphisms of *Avpr-1a* gene are also associated in ASD (Kim et al., 2002; Wassink et al., 2004; Yirmiya et al., 2006). Taken together, these studies highlight the possible role of genetic variations of neuropeptide receptors in the development of ASD. This is in line with a large body of animal studies that have revealed the importance of both *Avpr* and *Oxtr* genes in the regulation of social behaviour (Lim and Young, 2006; Carter, 2007).

Finally, two studies suggest that systemic infusions of OXT reduce repetitive behaviour in ASD (Hollander et al., 2003) and improve emotion recognition in ASD (Hollander et al., 2007). Although these studies used systemic infusions of

OXT, giving rise to the above-mentioned concerns about the transmission of the peptide to the brain, the results are consistent with the effects reported after intranasal administration in healthy males (Domes et al., 2007b).

To summarize, there is increasing evidence that the *Oxtr* gene might be involved in the development of ASD. Furthermore, a number of studies show that the availability of OXT is associated with socio-cognitive functioning in ASD. It should be noted that there are also studies that link ASD to alterations of AVP and related neuropeptides, such as apelin (Momeni et al., 2005; Boso et al., 2007).

Obsessive-compulsive disorder

According to DSM-IV, recurrent, intrusive thoughts and fears of danger or contamination, and compulsive behaviours (e.g. excessive hand-washing) or cognitions for relieving anxiety are the most prominent symptoms of OCD. Based on the mnemonic effects of OXT and AVP (see above) and the possible role of both peptides in self-grooming behaviour in animals (Pedersen et al., 1988; Lumley et al., 2001), it has been put forward that OCD symptoms might be associated with alterations in central neuropeptide functioning (cf. Leckman et al., 1994a). This idea stimulated several clinical studies on OXT and AVP in OCD, which we will review in the following section.

Regarding CSF and plasma levels of AVP, adult OCD patients showed elevated basal CSF levels of AVP and increased secretion of AVP into the plasma in response to hypertonic saline administration (Altemus et al., 1992). In contrast, Leckman et al. (1994b) reported normal CSF concentrations of AVP for OCD patients. In a study with children, CSF AVP concentration and the AVP/OXT ratio were negatively correlated with OCD symptom severity (Swedo et al., 1992), which might suggest developmental changes in AVP in patients with OCD.

Studies investigating CSF levels of OXT found enhanced CSF levels of OXT in children and adolescents with OCD compared with other anxiety disorders and healthy controls (Swedo et al., 1992), and in adults with non-tic-related OCD compared to tic-related OCD, Tourette Syndrome, and healthy controls (Leckman et al., 1994b). In addition, Leckman et al. (1994b) found a strong correlation between the severity of compulsion (measured with the Yale–Brown Obsessive Compulsive Scale) and CSF OXT in non-tic-related OCD. Altemus et al. (1999) were not able to confirm the finding of enhanced OXT levels in OCD.

Although an early case study reported symptomatic improvement in OCD patients treated with intranasal OXT (Ansseau et al., 1987), subsequent controlled studies did not confirm therapeutic effects of systemic (Charles et al., 1989) or intranasal administration (den Boer and Westenberg, 1992; Salzberg and Swedo, 1992; Epperson et al., 1996a, b) of OXT in OCD. These negative results might be attributed to the commonly low statistical power due to insufficient sample sizes (den Boer and Westenberg, 1992; Salzberg and Swedo, 1992; Epperson et al., 1996a, b), the short-term treatment (Salzberg and Swedo, 1992; Epperson et al., 1996a, b) or to low doses of treatment (den Boer and Westenberg, 1992; Salzberg and Swedo, 1992). Another study revealed significant changes in CSF neuropeptide concentrations following long-term clomipramine treatment of child and adolescent OCD patients: AVP decreased (among corticotropin-releasing hormone and somatostatin), whereas OXT increased in response to clomipramine treatment (Altemus et al., 1994).

Taken together, the findings on the role of OXT and AVP in OCD are inconsistent. Thus, further research is needed to elucidate the potential role of OXT and AVP on compulsive behaviour and ruminative, obsessional thoughts and fears in OCD.

Borderline personality disorder and early trauma

BPD is characterized by a pervasive pattern of instability in affect and interpersonal relationships as well as by (auto-)aggressive behaviours (Lieb et al., 2004). In particular, BPD has been associated with excessive socio-affective vigilance and enhanced reactivity to emotional and social stimuli (Herpertz et al., 1997). Hypervigilance to emotionally laden social stimuli is further confirmed by studies showing enhanced amygdala reactivity to negative scenes (Herpertz et al., 2001), negative facial expressions (Minzenberg et al., 2007), and

even to faces of neutral valence (Donegan et al., 2003). Furthermore, BPD patients have been described as hypersensitive to social signals, sometimes misinterpreting ambiguous subtle social cues in terms of a negativity bias (Wagner and Linehan, 1999), particularly towards the perception of anger (Domes et al., 2008).

Some researchers have reviewed the role of attachment theory for understanding BPD. According to these reviews, BPD is best characterized by unresolved, preoccupied and fearful types of attachment (Agrawal et al., 2004; Levy, 2005). The insecure attachment style exhibited by many BPD patients may be the basis for the fundamental distrust that BPD patients report with regard to their social relationships. Thus, alterations in the OXT/AVP system have been considered as a possible factor in the pathogenesis in disturbed adult attachment (Carter, 1998). Given the high prevalence of severe childhood trauma and neglect in BPD (Lieb et al., 2004), it has been speculated that early stress interferes with the developing neuropeptide system and alters receptor binding of OXT and AVP, thereby promoting the development of severe attachment disorders (Carter, 2003).

In line with this notion, Fries et al. (2005) found an association between reduced early physical and emotional contact and basal levels of plasma AVP. In this naturalistic study, early neglect had no effect on basal levels of OXT, but rather impaired the increase of peripheral OXT triggered by a mother–infant interaction (Fries et al., 2005). In addition, Meinlschmidt and Heim (2007) showed that a single dose of 24 IU intranasal OXT reduces salivary cortisol concentrations in healthy men with early parental separation in comparison with healthy control subjects. Thus, early neglect seems to impair the central regulation of peptide release and/or synthesis in social interaction.

Conclusion

Over the last decades, animal models have achieved enormous insights into how neuropeptides contribute to the regulation of social behaviour. We have reviewed a growing body of evidence from recent human studies indicating that the basic effects of OXT and AVP on social behaviour from animal research may also be applicable to human social interaction. Although the translation of behavioural and neurobiological findings from animal studies to humans generally bears the risk of drawing oversimplified parallels between rodents and humans, the findings to date are encouraging in terms of providing a better understanding of the neuroendocrine mechanisms of human social behaviour. Moreover, these translational findings suggest that OXT and AVP may play an important role in the etiology and treatment of a number of clinical disorders involving social deficits and disrupted attachment.

With regard to the role of OXT in human social behaviour, the main findings can be summarized as follows: (1) OXT is associated with the regulation of the behavioural and endocrine stress response, that is OXT is released in response to socially relevant challenges and attenuates the endocrine and autonomic responses to stress. (2) OXT is released in response to positive social interactions, such as social support or social proximity, thus possibly representing a mediator for the well-known stress-protective effects of social support. (3) The neural correlate for the anxiolytic effects of OXT has been suspected in limbic areas, in particular in the amygdala. OXT has been found to attenuate amygdala reactivity to emotional and social stimuli and to reduce brainstem activity, which is associated with autonomic arousal. (4) OXT has been found to promote social cognition and the interpretation of social signals, possibly representing an enhanced readiness to show social approach behaviour and empathy. (5) Finally, there is initial evidence that the central OXT system is altered in several mental disorders that are characterized by severe social disturbances, such as ASDs, OCD, personality disorders, and following early trauma. Although the role of OXT and other neuropeptides is not yet clear in terms of the etiology of these clinical disorders, there is preliminary evidence suggesting that genetic alterations of neuropeptide receptors and developmental challenges (e.g. early adverse experience) interact in the etiology and development of these disorders.

Although most of the studies conducted thus far on human social behaviour have focused on OXT, initial studies on AVP also suggest behavioural effects similar to those found in animal research. Specifically, central AVP has been shown to influence social communication in a sex-specific manner, promoting agonistic facial responses towards same-sex faces in men but affiliative responses in women.

Despite the progress of animal and human research on behaviourally relevant effects of the neuropeptides OXT and AVP, there are still a number of unresolved issues. Most of the studies investigating the effects of OXT in humans have restricted their samples to male volunteers. Future studies should include both sexes to determine whether the sexual dimorphism in the behavioural effects of OXT and AVP known from several vertebrate classes (De Vries and Panzica, 2006) also holds for human behaviour. The detailed mechanism of brain penetration of OXT and AVP following different methods of administration and the relationship between plasma and central OXT and AVP (including possible crosstalks of these neuropeptides at their respective central receptors) is another area that warrants further investigation (McEwen, 2004). Besides recent advances made in identifying neural activity using fMRI, the development of specific radioactive labelling of neuropeptides in positron emission tomography will be needed to understand how OXT and AVP receptors are mapped in the human brain.

Finally, basic research in animals and humans has stimulated studies regarding the idea that neuropeptides might be a significant target for novel therapeutic approaches aimed at reducing social anxiety and increasing social abilities in several mental disorders that are characterized by social interaction pathology. In a recent review, Carter (2007) noted that ''Knowledge of natural ways to stimulate the release of endogenous OXT or to inhibit 'excess' AVP might be protective against the development of the features of ASD [autism spectrum disorder], perhaps even remediating the expression of ASD-like behaviours in later life'' (p. 180). As for the anxiogenic and aggression-related role of AVP, the development of selective V1a and V1b receptor antagonists, as known from animal studies (Griebel et al., 2005; Ferris et al., 2006), is a promising target for human neuropsychopharmacological research, in particular in the treatment of stress-related disorders and disorders with interpersonal violence such as anti-social personality disorder. Unfortunately, to date, there are no V1a or V1b receptor antagonists available for use in humans.

The currently most promising approach seems to be to increase the availability of OXT in the central nervous system by exogenous administration of the neuropeptide or selective agonists. Notably, there is initial evidence for the applicability of this approach using systemic infusions of OXT in autism (Hollander et al., 2003, 2007). Although these studies show good potential, further studies are needed to test the hypothesis that patients with mental disorders associated with severe social deficits benefit from a combination of psychotherapy and OXT administration. In general, OXT treatment is expected to improve the readiness to socially interact (e.g. in role-played simulations) and to facilitate more active and successful engagement in confronting feared social situations outside of the sessions. To this end, a clinical research program being carried out in our laboratory aimed at developing and evaluating new clinically relevant approaches for disorders with social deficits (especially in social phobia, ASD, BPD). More specifically, we are currently conducting controlled treatment trials to investigate the effects of intranasal OXT administration in combination with standardized cognitive-behavioural therapy programs in different mental disorders with social deficits. Figure 1 shows an integrative model of the interactions of anxiety and stress, social approach behaviour, and the oxytocinergic system, which also integrates the novel approach of a psychobiological therapy in psychopathological states. Considering the large number of patients suffering from disorders that are associated with social deficits, the development of specific psychobiological approaches combining effective psychological methods with synergizing OXT or OXT agonist administration constitutes a primary challenge in interdisciplinary research on the treatment of mental and developmental disorders.

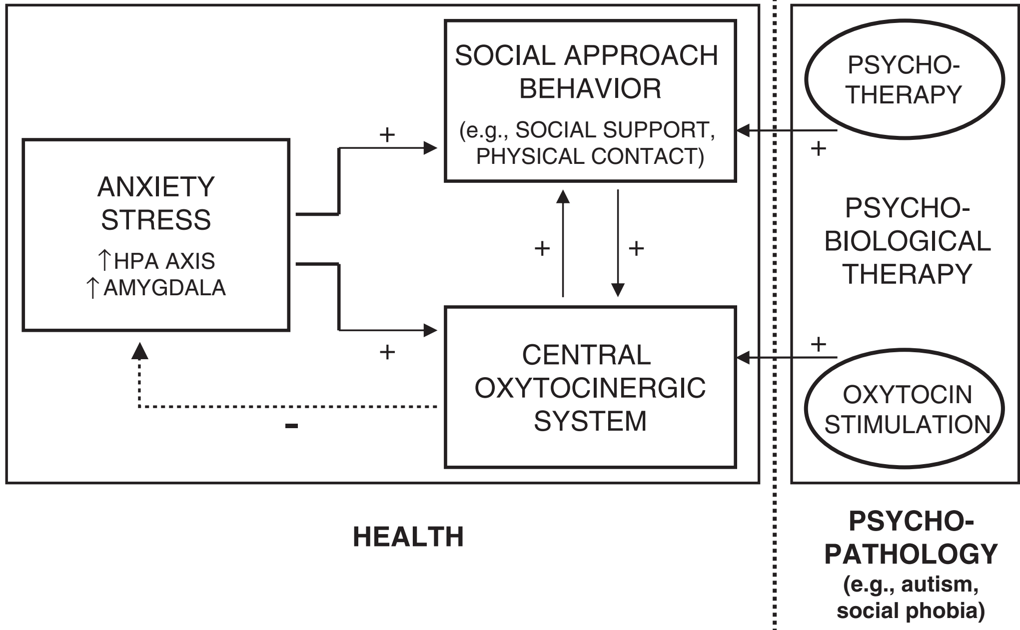

Fig. 1. Interactions between anxiety and stress, social approach behaviour, and the oxytocinergic system. Anxiety and stress encourage social approach behaviour and stimulate OXT release in healthy individuals. Different kinds of positive social interaction (e.g. physical contact) are associated with OXT release, and in turn, OXT promotes social approach behaviour. As OXT reduces HPA axis responses and limbic reactivity (especially amygdala) to social stressors, the neuropeptide plays an important role as an underlying neurobiological mechanism for the anxiolytic/stress-protective effects of positive social interaction. In mental and developmental disorders that are associated with severe deficits in social interactions (e.g. autism, social phobia, BPD), novel therapeutic approaches combining effective psychotherapy methods with OXT or OXT agonist administration offer the opportunity to develop a "psychobiological therapy".

Acknowledgements

This work was supported by grants from the Swiss National Science Foundation (SNSF PP001-114788) and the Research Priority Program "Foundations of Human Social Behavior" at the University of Zurich (to M. Heinrichs), and by a grant of the German Research Foundation (DFG Do1312/1-1) (to G. Domes).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 29

Chronic stress plasticity in the hypothalamic paraventricular nucleus

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Abstract: Proper integration and execution of the physiological stress response is essential for maintaining homoeostasis. Stress responses are controlled in large part by the paraventricular nucleus (PVN) of the hypothalamus, which contains three functionally distinct neural populations that modulate multiple stress effectors: (1) hypophysiotrophic PVN neurons that directly control the activity of the hypothalamic-pituitary-adrenocortical (HPA) axis; (2) magnocellular neurons and their secreted neurohypophysial peptides; and (3) brainstem and spinal cord projecting neurons that regulate autonomic function. Evidence for activation of PVN neurons during acute stress exposure demonstrates extensive involvement of all three effector systems. In addition, all PVN regions appear to participate in chronic stress responses. Within the hypophysiotrophic neurons, chronic stress leads to enhanced expression of secreted products, reduced expression of glucocorticoid receptor and GABA receptor subunits and enhanced glutamate receptor expression. In addition, there is evidence for chronic stress-induced morphological plasticity in these neurons, with chronic drive causing changes in cell size and altered GABAergic and glutamatergic innervation. The response of the magnocellular system varies with different chronic exposure paradigms, with changes in neurohypophysial peptide gene expression, peptide secretion and morphology seen primarily after intense stress exposure. The preautonomic cell groups are less well studied, but are likely to be associated with chronic stress-induced changes in cardiovascular function. Overall, the PVN is uniquely situated to coordinate responses of multiple stress effector systems in the face of prolonged stimulation, and likely plays a role in both adaptation and pathology associated with chronic stress.

Keywords: vasopressin; corticotropin-releasing hormone; magnocellular; parvocellular; preautonomic

The paraventricular nucleus (PVN) of the hypothalamus is a critical node for regulation and orchestration of physiological stress responses. Neurons resident in the PVN control three major physiological effector pathways: the hypothalamo-pituitary-adrenocortical (HPA) axis, through neurons in the dorsomedial parvocellular (PVNmpd) and anterior parvocellular (PVNap) divisions of the nucleus; neurohypophysial peptide signals, via magnocellular vasopressin (AVP) and oxytocin (OXT) neurons in the anterior (PVNam), medial (PVNmm) and posterior magnocellular (PVNpm) cell groups; and autonomic regulation, using brainstem and spinal cord projecting neurons in the lateral parvocellular (PVNlp), dorsal parvocellular (PVNdp) and ventromedial parvocellular (PVNmpv) divisions (Swanson and Sawchenko, 1983) (Fig. 1). The intermingling of

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DOI: 10.1016/S0079-6123(08)00429-9

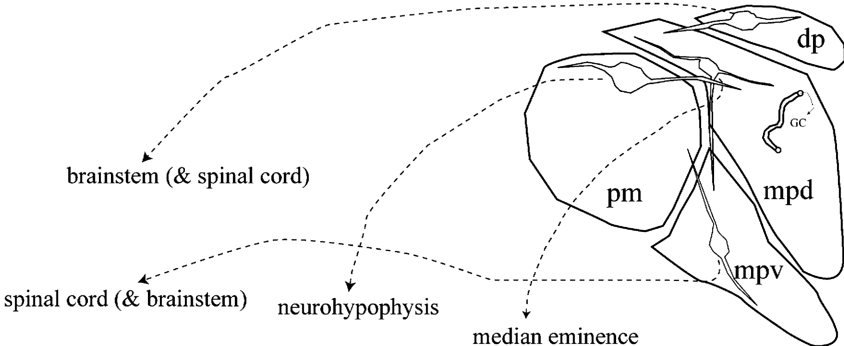


Fig. 1. Schematic of the organization of the hypothalamic paraventricular nucleus. Neurons in the dorsal parvocellular (dp) and ventral division of the medial parvocellular regions (mpv) project primarily to brainstem and spinal cord regions associated with autonomic control. Neurons present in the dorsal division of the medial parvocellular zone (mpd) project primarily to the median eminence and control ACTH release. The posterior magnocellular (pm) subdivision sends projections to the neurohypophysis. Note that the dendrites of neurons localized in all three regions ramify across subdivisions, allowing for intranuclear communication.

three physiologically distinct signalling systems places the PVN at the crossroads for integration of diverse outputs in a coordinated and inter-regulated fashion.

Regulation of the HPA axis is perhaps the most clear-cut stress integrative role of the PVN. Neurons in the PVNmpd express corticotropin-releasing hormone (CRH), which is required for activation of pituitary ACTH release (Antoni, 1986). Parvocellular CRH neurons also express numerous co-secreted peptides as well as glutamate (Swanson, 1991; Ziegler et al., 2002), indicating that multiple factors may be involved in parvocellular signalling. Importantly, AVP is among the peptides co-localized with CRH in PVNmpd neurons, and synergizes with CRH to enhance ACTH release at the level of corticotrope (Gillies et al., 1982).

The magnocellular neurosecretory system also plays a role in stress regulation. There is a substantial body of evidence to suggest that OXT can be released into the systemic circulation by acute stressors (Jezova et al., 1995; Wotjak et al., 1998). Peripheral release of AVP is more controversial, and does not occur following many stressors (Husain et al. 1979; Jezova et al., 1995; Wotjak et al., 1998). However, both peptides are released from dendrites in the supraoptic nucleus (SON) following stress (Wotjak et al., 1998) and appear to diffuse to limbic brain sites (Ludwig and Leng, 2006), where they have the capacity to influence behavioural stress responses.

Vasopressin is implicated in amplification of pituitary ACTH secretion (Gillies et al., 1982). There is also some evidence for an ACTH-potentiating action of OXT (Gibbs et al., 1984). Both peptides are released into the hypophysial portal blood, supporting the notion that they have access to the anterior pituitary (Gibbs et al., 1984; Plotsky and Sawchenko, 1987). There are data to suggest release of peptide from magnocellular axons of passage in the median eminence (Holmes et al., 1986), which may account for the portal blood OXT and perhaps AVP. Thus, the magnocellular system is positioned to play a role in control of HPA axis responsivity (Engelmann et al., 2004).

The magnocellular system may also participate in immune responses to stress. Cytokine-like effects have been ascribed to both AVP and OXT (Kovacs, 2002), and neurointermediate pituitary lobectomy alters humoral and cell-mediated immune responses (Quintanar-Stephano et al., 2004). Both AVP and OXT are capable of replacing the IL-2 requirement for the facilitation of interferon-γ from immune cells (Johnson et al., 1982). In addition to the 'cytokine-like' effects of the classic neurohypophysial peptides, several cytokines (i.e. IL-1β (Watt and Hobbs, 2000), IL-1ra (van Dam et al., 1998) and IL-6 (Ghorbel et al., 2003)) are

expressed in the magnocellular neurons of the PVN and SON. Both IL-6 and IL-1β are found in the posterior pituitary and median eminence (Watt and Hobbs, 2000; Jankord et al., 2007) and can be released in response to dehydration (Ghorbel et al., 2003; Summy-Long et al., 2006). The ability to store and secrete cytokines and other immune-modulating factors allows the magnocellular system to influence the organismal response to immune challenge. In combination with the output from the HPA axis and sympathetic nervous system, the secreted cytokine and cytokine-like products of the neurohypophyseal neurons may affect responses to immune challenge and thus participate in a 'neuroimmune' stress response.

Brainstem and spinal cord-projecting neurons represent the final major cellular subpopulation in the PVN. These neurons express a number of neuropeptides, with a substantial population being oxytocinergic (Hallbeck et al., 2001). The projection pathways suggest a role in autonomic regulation, and there is evidence to support a role for these PVN neurons in sympathetic activation consequent to stress. Viral tracing studies confirm that populations of neurons within the PVN have oligosynaptic connections with both sympathetic and parasympathetic targets (Kalsbeek et al., 2004), suggesting that the role of the PVN in autonomic integration may involve regulation of both systems.

It is important to note that there is substantial opportunity for intranuclear communication within the PVN. There is strong evidence for dendritic release in the PVN and magnocellular SON (Landgraf and Neumann, 2004; Ludwig and Leng, 2006), implying that activation of one compartment or neuronal cell type can have a marked influence on adjoining, functionally distinct cell populations.

Activation of PVN neurons by acute stressors

The central role of the PVNmpd in HPA activation is underscored by numerous studies documenting induction of immediate early gene expression (Fos, NGFI-B) following exposure to stressors, consistent with stimulation-induced transcriptional activation (Kovacs and Sawchenko, 1996). This conclusion is further supported by studies documenting rapid phosphorylation of cyclic AMP response element binding protein (CREB) and mitogen activated protein (MAP) kinase following stressors (Kovacs and Sawchenko, 1995; Khan and Watts, 2004). Stressor exposure induces transcription of CRH as well as AVP genes in the parvocellular PVN, and at later time points, increased CRH and AVP mRNA pools (Herman et al., 1992; Herman, 1995; Kovacs and Sawchenko, 1995). Moreover, acute stress exposure results in decreased CRH content in the median eminence (Chappell et al., 1986; Feldman and Weidenfeld, 1998), consistent with release, and directly increases portal blood levels of CRH and AVP (as well as OXT) (Gibbs et al., 1984; Plotsky and Sawchenko, 1987; Romero et al., 1993).

Activation of magnocellular neurons, as reflected by immediate early gene expression, is also induced by acute stress. In general, the threshold for activation of OXT neurons appears to be lower than that of AVP cells. For example, exposure to immobilization causes release of OXT, but not AVP into the peripheral circulation (Jezova et al., 1995), and greater numbers of OXT neurons are activated by stressors such as lipopolysaccharide (Matsunaga et al., 2000), interleukin 1-beta (Ericsson et al., 1994) or immobilization (Jezova et al., 1995). Fos activation of magnocellular AVP neurons is observed following hypertonic saline exposure, haemorrhage and hypoxia (Sharp et al., 1991; Thrivikraman et al., 2000; Figueiredo et al., 2003), suggesting that these neurons are responding to disruption of fluid and electrolyte balance, rather than stress per se. Fos expression is also observed in OXT cells under these conditions (Giovannelli et al., 1992; Ding et al., 1994).

Other stressors, such as restraint (a considerably milder stimulus than aforementioned immobilization), swim, and novelty, have less clear-cut actions on the magnocellular system (Cullinan et al., 1995; Emmert and Herman, 1999; Figueiredo et al., 2003). Notably, time course studies suggest that induction of *c-fos* mRNA occurs at relatively long post-stress intervals (Cullinan et al., 1995), suggesting that under these conditions, magnocellular neurons are responding to the physiologic

consequences of the stress response, rather than the stressor per se.

Fos activation in PVN preautonomic cell groups is also seen in response to acute stressors. Several combined tract-tracing and Fos mapping studies indicate that brainstem- and spinal cord-projecting PVN cell groups are activated by water deprivation, insulin, lipopolysaccharide and acute restraint (Zhang et al., 2000; Carrasco et al., 2001; Stocker et al., 2004; Radley et al., 2006), suggesting that like the parvocellular CRH system, the preautonomic PVN is responsive to a wide range of stressors. The response of the preautonomic system to interleukin 1-beta is prolonged relative to that of the PVNmpd (Ericsson et al., 1994), suggesting that this system may respond to some stimuli with an extended time course.

Chronic stress: cellular responses of PVN neurons

Chronic stress exposure produces episodic and cumulative increases in circulating corticosteroids and catecholamines. Given the impact of single stressors on PVN neurons, it is not surprising that chronic exposure results in pronounced changes in gene expression, peptide levels and releasable peptide pools. Within the PVNmpd, a variety of chronic stress paradigms, such as footshock, immobilization, chronic unpredictable stress, social subordination and social defeat, cause a reliable increase in CRH mRNA expression (Imaki et al., 1991; Herman et al., 1995; Makino et al., 1995; Albeck et al., 1997; Keeney et al., 2006). Importantly, CRH mRNA induction by repeated immobilization is observed across multiple rat strains (Gomez et al., 1996), indicating that this is a consistent result of chronic stress. In addition to CRH, chronic stress exposure increases expression of AVP mRNA in parvocellular neurons, both in terms of number of detectable cells and cellular grain density (Herman et al., 1995; Makino et al., 1995; Albeck et al., 1997). In addition, AVP peptide co-localization with CRH is increased in the median eminence following repeated immobilization (de Goeij et al., 1991). The latter observation predicts increased AVP activity at the level of the anterior pituitary, although this has yet to be definitively proven.

The changes discussed above are observed in most, but not all stress paradigms. Notably, adjuvant arthritis decreases CRH mRNA levels, despite the presence of elevated corticosterone (Harbuz et al., 1992). However, parvocellular AVP levels are markedly increased in arthritic rats, suggesting that this peptide may take on a larger role in mediating ACTH release during the inflammatory process (Shanks et al., 1998).

Activation of magnocellular neurons is observed in some, but not all chronic stress paradigms. Chronic stressors that involve modulation of fluid and electrolyte balance (e.g. hypertonic saline) reliably increase expression of both AVP and OXT in magnocellular neurons (Kiss and Aguilera, 1993; Glasgow et al., 2000). Neural activation is accompanied by increased secretion of peptide into the systemic circulation (Kiss and Aguilera, 1993). Activation of AVP neurons may be related to altered fluid and electrolyte homoeostasis in this model. Other models, including chronic variable stress (Herman et al., 1995), chronic social stress (Albeck et al., 1997), do not result in increased AVP mRNA expression, consistent with the latter interpretation. The significance of elevated magnocellular OXT following chronic hypertonic saline is unclear, and surprisingly little is known about regulation of OXT in other chronic stress paradigms.

Magnocellular neurons may play an important role in behavioural responses to chronic stress. Recent studies from Landgraf et al. (2007) indicate that elevations in paraventricular AVP are correlated with high anxiety behaviour in both rat and mouse strains bred for low or high anxiety. The effects of elevated AVP on behaviour appear to be related to dendritic release (Ludwig and Leng, 2006), raising the possibility that magnocellular AVP may affect multiple domains of PVN action.

In addition to affecting production of PVN effector molecules, chronic stress also causes marked changes in expression of PVN neural signalling molecules. Notably, chronic unpredictable stress and repeated immobilization both elicit

down-regulation of glucocorticoid receptor (GR) mRNA expression in the medial parvocellular PVN (Herman et al., 1995; Makino et al., 1995). The PVN GR is known to be involved in inhibition of the HPA axis (Kovacs et al., 1986; Kovacs and Makara, 1988). By virtue of its role as a ligand-gated transcription factor, decrements in GR expression may precipitate broad changes in gene expression in the parvocellular PVN. Indeed, the loss of GR may be connected with up-regulation of both CRH and AVP mRNAs in the PVNmpd (Herman et al., 1995).

Expression of co-localized neuropeptide species is also modulated by chronic stress. Chronic hypertonic saline administration or morphine withdrawal lead to elevated proenkephalin expression in both parvocellular and magnocellular PVN neurons, as well as magnocellular neurons in the SON (Harbuz et al., 1991; Young and Lightman, 1992).

Chronic stress also causes marked changes in expression of a number of PVN neurotransmitter receptors. Notably, the beta-1 and beta-2 subunits of the GABA-A receptor are down-regulated in the PVNmpd (but not PVNpm) by chronic unpredictable stress (Cullinan, 2000), consistent with reduced efficacy of GABA signalling (and hence, inhibition) specifically in parvocellular PVN neurons. These data are supported by electrophysiological studies documenting reduced GABA signalling in PVN slices from chronically stressed rats (Verkuyl et al., 2004).

In contrast, excitatory processes appear to be enhanced in PVNmpd neurons. Chronic variable stress causes marked increases in expression of the GluR5 subunit of the kainate-preferring glutamate receptor in the PVNmpd, consistent with enhanced capacity for glutamate signalling through non-NMDA channels (Fig. 2). Expression of NMDA-R1 and NMDA-R2A receptor subunits are not altered by chronic stress. However, the NMDA-R2B receptor is significantly down-regulated (Ziegler et al., 2005). Receptors containing the NMDA-R2B subunit are less permeable to calcium then those containing NMDA-R2A (c.f. Loftis and Janowsky, 2003), suggesting that decreased expression of this receptor subunit may enhance NMDA-mediated calcium currents, also

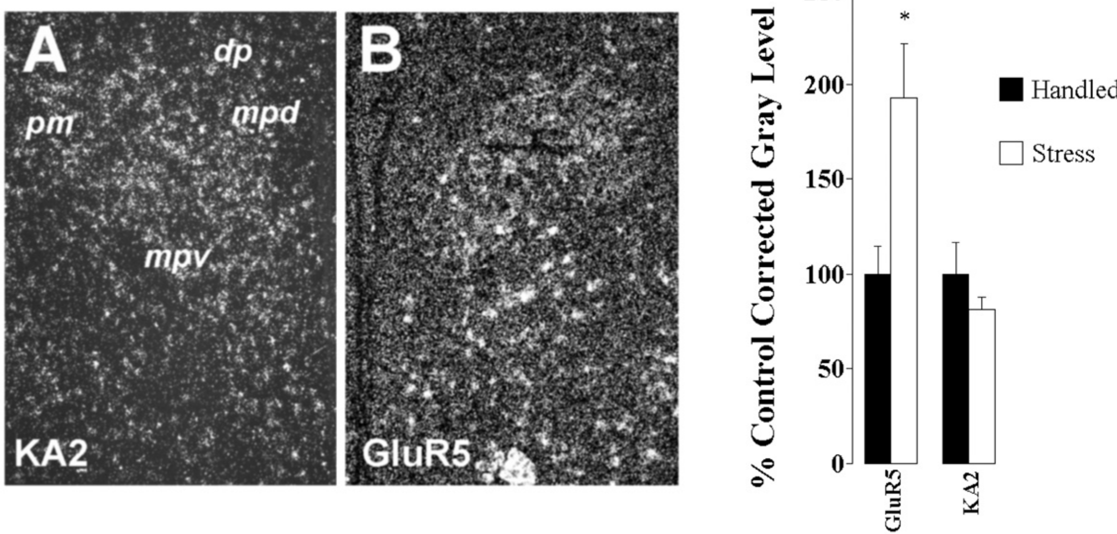


Fig. 2. Distribution and regulation of KA2 and GluR5 receptor subunits in the PVN. As noted in A, KA2 mRNA is expressed in all subregions of the PVN (see Fig. 1 for abbreviations) GluR5 mRNA expression is particularly enriched in the PVNmpd. Semi-quantitative analysis of KA2 and GluR5 subunit mRNA expression in the PVN. Exposure to CVS causes a marked up-regulation of GluR5 expression in the PVN, whereas KA2 mRNA levels are unchanged.

consistent with enhanced glutamate signalling. In addition, there is evidence that the alpha-1B adrenergic receptor is up-regulated under conditions of high HPA axis drive (adrenalectomy) (Day et al., 1999). Coupled with studies showing enhancement of acute stress-induced PVN norepinephrine release following chronic cold exposure (Ma and Morilak, 2005), these data suggest the chronic stress enhances stress-excitatory norepinephrine signalling.

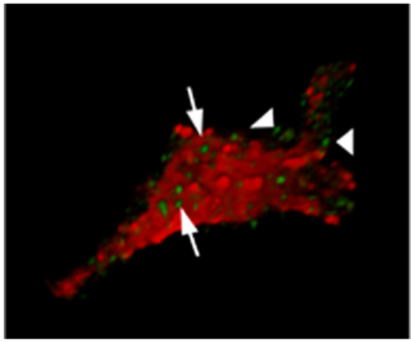
The influence of chronic stress on regulation of preautonomic PVN neurons remains to be clearly established. There is evidence for alterations in preautonomic PVN regions in experimental models of chronic heart failure (Patel and Zhang, 1996; Vahid-Ansari and Leenen, 1998). However, in this model it is difficult to conclude if the activation pattern is due to stress or reflex compensation for autonomic dysfunction. Nonetheless, chronic stress exposure has deleterious effects on cardiovascular regulation (Grippo et al., 2002), and further study is clearly needed to determine the contribution of preautonomic PVN neurons to this process.

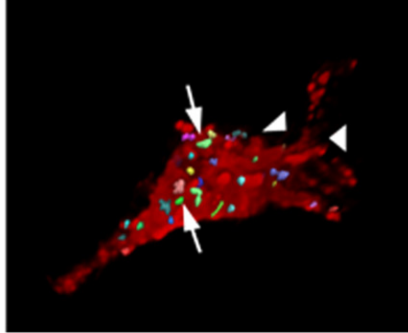
Chronic stress: morphological plasticity in PVN

There is evidence that structural changes in magnocellular neurons are a consequence of prolonged neuronal activation. Electron micrographic studies present evidence for cellular hypertrophy and glial retraction in the SON of rats during prolonged stimulation (in the case of AVP neurons, salt loading/dehydration; in the case of OXT neurons, lactation) (Hatton, 1997). Lactation increases excitatory and inhibitory synaptic contacts onto both AVP and OXT neurons (Theodosis and Poulain, 1993; Theodosis, 2002), accompanied by increases in norepinephrine-containing terminals onto OXT neurons (Michaloudi et al., 1997). In the vasopressinergic system, salt loading causes increased numbers of GABA and glutamate terminal appositions on magnocellular neurons of the SON (Mueller et al., 2005). However, there was a pronounced decrease in norepinephrine innervation of AVP neurons, suggesting that regulation of synaptic plasticity may be stressor-specific (Mueller et al., 2005). Finally, altered dendritic branching is also observed during lactation, with oxytocinergic neurons exhibiting retraction and vasopressinergic dendrites showing extension (Stern and Armstrong, 1998). The dendritic changes are consistent with activity-dependent remodelling in the magnocellular system, and further indicate that magnocellular neuronal phenotypes have distinctive growth responses to stimulation.

The studies cited above involve stimulation at extreme levels of physiologic drive. However, even milder stressors, i.e. repeated restraint, can increase cell size and incidence of multiple synapses on magnocellular SON neurons (Miyata et al., 1994), suggesting that chronic stress may also drive changes in magnocellular synaptology. For this reason, the effect of chronic stress on hypophysiotrophic and preautonomic cell populations is currently a topic of considerable interest. Recently, our group tested the ability of chronic stimulation to alter the innervation of parvocellular PVN neurons. To test this hypothesis, we assessed glutamate innervation of parvocellular PVN neurons following adrenalectomy , which produces pronounced hyperactivation of PVN CRH neurons (Sawchenko, 1987). Our data are summarized in Fig. 3. Using three-dimensional reconstructions of confocal stacks double-stained for CRH and vesicular glutamate transporter 2 (a glutamate marker), we were able to demonstrate enhanced glutamate innervation of CRH neurons following chronic central stimulation of the PVNmpd. Subsequent studies in chronically stressed animals suggest that the same reorganization occurs in response to chronic variable stress (Flak, Ostrander, Mueller, and Herman, unpublished observations).

Notably, administration of IL-1 or amphetamine produces long-lasting sensitization of HPA axis responses that correlates with a reduction of dopamine-beta-hydroxylase (DBH) immunoreactivity in the dorsal and medial parvocellular subdivisions of the PVN 3 weeks after presentation of the stressor (Jansen et al., 2003). Thus, it is possible that even short-term stress exposure can result in substantial alterations in PVN innervation.





VGlut2 Bouton Appositions on CRH neurons: 3-D Analysis

	Boutons/10000um^3	Bouton vol/10000um^3	Bouton volume (um^3)
Sham	17.9 ± 2.5	18.6 ± 5.1	0.9 ±0.2
ADX	28.1 ± 3.6*	50.1 ± 10.9*	1.7 ± 0.2*

Fig. 3. 3-D rendering of confocal images: CRH and VGLUT2. Left: cropped, 3-D rendering of a single CRH cell (red) amid green VGlut2 puncta, indicative of glutamatergic terminals. Right: processed image of the same cell, with multi-coloured boutons indicating those showing significant overlap between red and green fluorescence signal. Arrows indicate examples of boutons that 'contact' the CRH neuron, as determined by the 'overlap' algorithm in Volocity 2; boutons that do not contact (arrowheads) do not register as overlapping entities. The volumes of positive elements in the cell and bouton channels were measured independently, using the classification features; overlap was determined using the co-localization feature, which determines incidence and volume of boutons contacting the immunolabelled cell. Preliminary analysis of ADX data using 6-cells/group (2 each from 3 animals/group) revealed a 56% increase in VGlut2 bouton counts/cell surface area using this method. In addition, ADX animals showed an increase in contact bouton overlap area and in overall bouton size. (See Color Plate 29.3 in color plate section.)

Neuroplasticity and PVN responses to chronic stress

The data reviewed above provides ample evidence for multifaceted neuroplastic responses in the PVN following chronic stress. Chronic stress-induced changes in PVN function are summarized in Fig. 4. First, chronic stress changes the cocktail of secretagogues synthesized and released by the PVNmpd. Enhancement of parvocellular CRH and AVP are likely to lead to enhanced pituitary responsiveness to stress, and may account for the sustenance of corticosterone secretion during chronic stress and perhaps in stress-related pathologies. Second, the loss of local GRs stands to decrease local glucocorticoid feedback and enhance cellular responsivity. Chronic stress-induced increases in parvocellular CRH and AVP may be fuelled in part by the loss of glucocorticoid feedback in the PVN. Third, chronic stress causes changes in the receptor configuration in the PVNmpd, suggestive of reduced numbers of functional GABA-A receptors and increased glutamatergic and perhaps noradrenergic signalling. The aggregate response to receptor changes would be predicted to once again enhance excitability and increase stress responsiveness. Finally, recent data suggest that drive of the parvocellular system by adrenalectomy or chronic stress enhances glutamate innervation to CRH neurons. This change also predicts enhanced activational capacity of the HPA axis. Overall, it appears that chronic drive of the parvocellular system leads to a multiplicity of neuroplastic changes that, in toto, result in an HPA axis that is poised to hyperrespond to incoming stimuli.

The impact of chronic stress on magnocellular and preautonomic components are less well understood. Certainly, stress can enhance magnocellular production of neurohypophysial peptides and increase cell size (see above), but the overall significance of these changes with respect to stress signalling are only now being studied. One notable factor that emerges from the literature is an apparent connection between stressor modality and intensity. Stress activation of magnocellular neurons occurs under conditions of homoeostatic

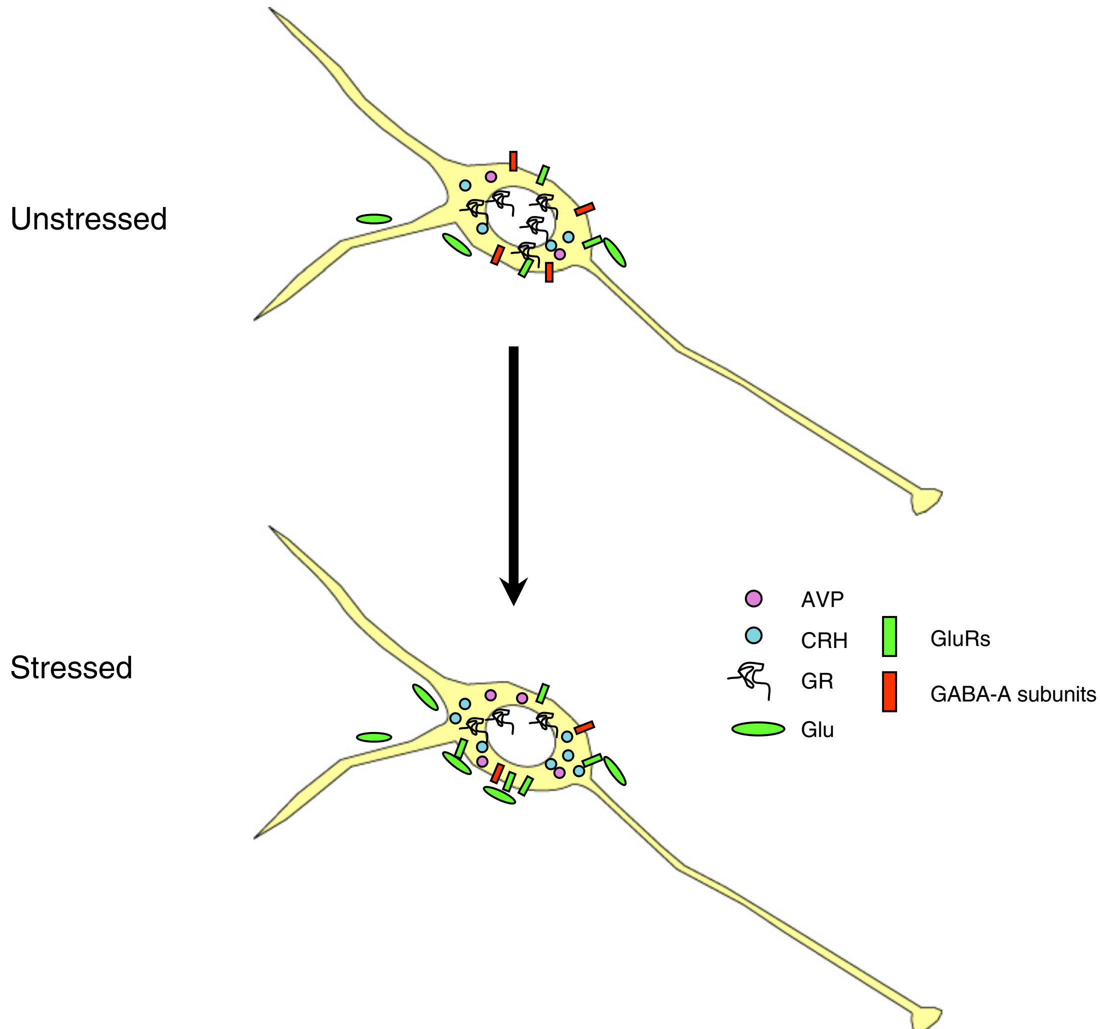


Fig. 4. Schematic diagram of multiple neuroplastic changes in the PVNmpd under conditions of chronic drive. Following chronic stress, there is increased expression of ACTH secretagogues CRH and AVP. At the same time, expression of the glucocorticoid receptor (GR) is down-regulated, suggesting loss of feedback capacity. Expression of the GABA-A receptor subunits beta-1 and beta-2 is decreased, consistent with reduced GABAergic inhibition. In contrast, expression of the kainate preferring GluR5 subunit mRNA is increased, suggesting increased glutamate signalling via non-NMDA receptors. Finally, glutamatergic innervation of CRH neurons is markedly increased following chronic drive by adrenalectomy, suggesting the potential for stress-induced synaptic plasticity. (See Color Plate 29.4 in color plate section.)

stimuli (hypoxia, haemorrhage, salt loading), suggesting that the magnocellular system may be preferentially targeted by homoeostatic response pathways. Notably, the magnocellular PVN and SON receive preferential input from the A1 and C1 catecholamine groups in the ventrolateral medulla (Cunningham and Sawchenko, 1988; Cunningham et al., 1990), which are thought to be primary effectors of sympathetic outflow. Thus, engagement of the magnocellular system may be keyed to ascending input signalling disruptions of the internal milieu. In addition, homoeostatic stressors are typically quite intense, in terms of their ability to activate other stress pathways (such as the HPA

axis). In this regard, it is important to note that the magnocellular system can also be activated by prolonged or intense stressors that are not of homoeostatic origin (e.g. prolonged restraint). Thus, it is possible that the magnocellular system is recruited at high threshold stimulation.

The nature of the signal provided by the magnocellular system is also worthy of consideration. Vasopressin has the capacity to interact with CRH to stimulate the HPA axis and has direct pressor actions that can assist in stress activation. However, OXT released within the PVN has marked anti-stress properties (Neumann et al., 2000). Moreover, numerous studies indicate that stress exposure disproportionately activates magnocellular OXT neurons (Jezova et al., 1995; Ericsson et al., 1997; Matsunaga et al., 2000), and that some acute regimens activate the magnocellular system at long post-stimulus time frames (Cullinan et al., 1995). Thus, magnocellular OXT and perhaps AVP may play a role in stress recovery, serving to dampen rather than promote stress responses.

Finally, stress plasticity in the preautonomic division is largely unexplored. Given clear influences of stress and stress-related affective states (i.e. depression) on cardiovascular disease, this is clearly an area deserving of additional attention.

In summary, it is evident that the PVN is a critical coordinator of the organismal stress response. The interconnections within the nucleus and the diversity of outputs suggest considerable potential for orchestration and sculpting of the physiological response to adversity. Long-term functional changes in excitability following chronic stress are consistent with an adaptation of the animal to periods of challenge (i.e. maintenance of response capacity in the face of chronic drive). Naturally, inappropriate drive of the PVN by aggregated psychogenic stimuli (perhaps those encountered by many in daily life) may underlie stress-related disease in vulnerable individuals, wherein stress responses are initiated and perpetuated in the absence of truly life-threatening stimuli. Thus, the PVN (and perhaps SON) may be a major part of the effector pathways responsible for stress-related disease, and hence a potential target for intervention or amelioration of these disorders.

Acknowledgements

This work was supported by NIH grants MH049698, MH069725 and MH069680.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 30

Keeping oxytocin neurons under control during stress in pregnancy

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Abstract: Oxytocin synthesised by magnocellular neurons in the supraoptic and paraventricular nuclei plays an important role in mammalian parturition. Accordingly, in late pregnant rats, oxytocin neurons are restrained from premature activation and stimulated oxytocin secretion is inhibited, preserving the expanded neurohypophysial oxytocin stores for parturition. A wide range of stressors stimulate oxytocin secretion in the rat. Some physical stressors, in particular immune challenge with systemic interleukin-1β (IL-1β, a cytokine that mimics infection) signal to magnocellular oxytocin neurons via brainstem noradrenergic neurons. Afferents relaying information from the uterus and birth canal also converge onto brainstem noradrenergic neurons and are robustly activated at parturition. Thus, quiescence of these inputs may be important in minimising the risk of preterm labour. Focussing on an immune challenge model (since infection is a major cause of preterm labour in women), we have found that the responsiveness of oxytocin neurons to IL-1β is markedly suppressed in late pregnancy. Here we discuss the mechanisms involved, which include induction of central inhibitory opioid tone by the progesterone neurosteroid metabolite, allopregnanolone, and act to prevent activation of oxytocin neurons by inappropriate stimuli at the end of pregnancy.

Keywords: allopregnanolone; endogenous opioids; interleukin-1β; noradrenaline; nucleus tractus solitarii; paraventricular nucleus; supraoptic nucleus

Introduction

Magnocellular oxytocin neurons in the supraoptic (SON) and paraventricular nuclei (PVN) project to the posterior pituitary. Oxytocin is secreted from the nerve terminals in the posterior pituitary in pulses during births and suckling, when the cell bodies discharge action potentials in high frequency coordinated bursts, which are important for driving parturition and essential for milk ejection (Russell et al., 2003). In both of these circumstances, oxytocin neurons are triggered to fire in bursts by afferent stimuli, from the contracting uterus and birth canal during parturition and from the suckled nipples during lactation (Russell et al., 2003). During both parturition and lactation, this afferent drive involves ascending spinal pathways projecting via A2 noradrenergic neurons in the nucleus tractus solitarii (NTS) and then to the magnocellular oxytocin neurons, forming the afferent limb of a positive feedback system (Russell et al., 2003). When the target tissue is especially sensitive to hormone action in such a system, activation of the positive feedback loop

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DOI: 10.1016/S0079-6123(08)00430-5

can potentially drive the system uncontrollably, until the stimulus is removed, either in the case of oxytocin secretion, by birth of the young at parturition or by satiation of the suckling young in lactation.

The myometrium and myoepithelial cells around the alveoli in the mammary gland are exquisitely sensitive to the actions of oxytocin at the end of pregnancy due to up-regulated oxytocin receptor expression (Soloff and Wieder, 1983; Larcher et al., 1995). Since in parturition the afferent limb of the positive feedback loop that stimulates magnocellular oxytocin neurons arises from the contracting uterus and birth canal, if the secretion of oxytocin from the maternal posterior pituitary is stimulated prematurely and the myometrium is sensitive to oxytocin, then activation of the positive feedback mechanism may precipitate preterm birth. The operation of such a mechanism in women is supported by the effectiveness of an oxytocin antagonist in preventing the progression of threatened preterm labour (Goodwin et al., 1994; Hussulein et al., 2007). Studies in the rat have shown central mechanisms that are activated during pregnancy to restrain oxytocin neurons from such premature activation. These mechanisms involve inhibition by endogenous opioids (Douglas et al., 1995) and by allopregnanolone (Russell and Brunton, 2006), a neurosteroid metabolite of progesterone (discussed below). Identification of these mechanisms has emerged from studies of oxytocin secretion and action during parturition (Leng et al., 1988; Antonijevic et al., 1995), and during late pregnancy where a range of stimuli that normally increase oxytocin secretion are less effective (Bull et al., 1994; Neumann et al., 1998; Brunton et al., 2006b). Several of these stimuli are stressors, since they also normally activate the hypothalamo-pituitary-adrenal (HPA) axis, and it is well established that in the rat oxytocin is secreted in response to a range of stressors (Lang et al., 1983; Neumann et al., 2001; Brunton et al., 2006b). The attenuated magnocellular oxytocin neuron responses to these stimuli is relevant to understanding the regulation of oxytocin secretion during parturition, and to the prevention of preterm stimulation of oxytocin secretion since physical stressors converge onto NTS A2 noradrenergic neurons (Luckman, 1992; Ericsson et al., 1994), as do afferents from the uterus and birth canal (Antonijevic et al., 1995; Luckman, 1995a; Meddle et al., 2000).

In addition to the advantageous action of the mechanisms that restrain oxytocin neurons in late pregnancy in preventing preterm stimulation of the myometrium, these mechanisms also permit the accumulation of an enlarged oxytocin store in the posterior pituitary, since the secretion of oxytocin in response to extraneous stimuli is decreased (Brunton et al., 2006b). Another consequence of reduced reactivity of the magnocellular oxytocin system relates to the role of centrally released oxytocin, probably from magnocellular neuron dendrites, in appetite regulation (Arletti et al., 1989). Oxytocin has anorectic actions (Verbalis et al., 1991; Douglas et al., 2007), and reduced central release of oxytocin in pregnancy is expected to contribute to increased appetite, which ensures provision of sufficient nutrients for growth of the foetuses, placentae and reproductive organs and for deposition of an adipose energy store for lactation.

In the rat, oxytocin neurons are osmosensitive, secreting oxytocin from the posterior pituitary in response to increased osmolarity; Na^+ excretion is consequently stimulated (Stricker and Verbalis, 1986). In late pregnancy, oxytocin neurons are also less responsive to a hyperosmotic stimulus; this reduced responsiveness also involves opioid inhibition (Bull et al., 1994). The reduced responsiveness of magnocellular oxytocin neurons to a modest hyperosmotic stimulus contributes a further factor that reduces oxytocin neuron activity in late pregnancy.

To summarise, for successful pregnancy and parturition, oxytocin neurons should (1) not be excited prematurely, (2) store oxytocin, and (3) be prepared to be excited by the appropriate signals at parturition. These demands are achieved by retention of the excitability of oxytocin neurons, but with restraining mechanisms on their inputs. Thus, at term, the stimulation of oxytocin secretion requires either (1) withdrawal of the restraining mechanisms, (2) powerful stimulation by afferent inputs from the contracting uterus and the birth canal (following the initiation of uterine

contractions at term by local intrauterine mechanisms) or (3) a combination of both.

The neurohypophysial oxytocin system and responses to stress

The HPA axis is the classical neuroendocrine stress response system; however, in addition to reproductive (i.e. uterine contractions and suckling) and osmotic stimuli, oxytocin neurons also respond to stressful stimuli. Stressors are broadly classified as either psychological (also emotional or processive) or physical (also physiological or systemic). Psychological stressors (e.g. restraint, noise, exposure to the elevated plus-maze) depend upon the individual's perception and are generally processed by forebrain limbic regions, whereas physical stressors (e.g. cold exposure, haemorrhage, immune challenge) do not rely on the individual's consciousness, pose an immediate threat and are processed via brainstem regions (for review, see Herman et al., 2003). While the role of the HPA axis during stress is well defined, the functional importance of oxytocin release in response to stress is not completely understood. Oxytocin released from magnocellular neurons can enter the portal blood to potentiate CRH-induced adrenocorticotrophic hormone (ACTH) secretion (Lang et al., 1983; Gibbs, 1986, 1985); thus, oxytocin may modulate HPA axis responses to stress (Engelmann et al., 2004).

In male and non-pregnant female rats, the neurohypophysial oxytocin system is stimulated by a wide range of stressors, including forced swimming (Lang et al., 1983), immobilisation (Lang et al., 1983), social defeat (Neumann et al., 2001) and systemic cholecystokinin (CCK, a satiety factor) (Douglas et al., 1995), hypertonic saline (Brimble et al., 1978) and interleukin-1β (IL-1β, a cytokine produced by activated macrophages in response to infection) (Naito et al., 1991; Brunton et al., 2006b), resulting in increased plasma concentrations of oxytocin. These stress-induced oxytocin responses are mediated centrally: neurosecretory magnocellular oxytocin neurons in the SON and PVN are activated (they express c-fos mRNA and Fos protein; indicators of neuronal activation) and increase their firing rate following exposure to stressors (Luckman, 1995b; Onaka et al., 1995b; Brunton et al., 2006b).

Brainstem noradrenergic inputs to magnocellular oxytocin neurons

Magnocellular neurons in the SON and PVN receive substantial innervation from brainstem noradrenergic neurons (Sawchenko and Swanson, 1981; Michaloudi et al., 1997) and α_1-adrenoceptors are expressed in the SON and PVN (Domyancic and Morilak, 1997). Neurons in the A2 cell group located in the NTS provide a direct excitatory input to oxytocin neurons, while neurons in the A1 cell group of the ventrolateral medulla (VLM) selectively excite vasopressin neurons via a direct projection to the SON/PVN, but also activate oxytocin neurons through an indirect pathway via the NTS (Day et al., 1984; Day and Sibbald, 1988; Raby and Renaud, 1989). Noradrenaline excites magnocellular oxytocin neurons and induces release of oxytocin from hypothalamic explants (Randle et al., 1986a, b). Administration of an α_1-adrenoreceptor agonist (Yamashita et al., 1987) or electrical stimulation of the A2 region (Day et al., 1984) increases the firing rate of magnocellular oxytocin neurons, an effect which can be abolished by neurotoxic lesions of the catecholamine inputs with 6-hydroxydopamine injections into the PVN (Day et al., 1984).

As mentioned above, stressful stimuli stimulate oxytocin neurons, and their activation by some stressors, particularly CCK and IL-1β, relies on brainstem noradrenergic neurons (Onaka et al., 1995a; Buller et al., 2001). IL-1β acts on IL-1 type 1 receptors expressed on the microvasculature supplying the brain (Ericsson et al., 1995) to stimulate cyclooxygenase and, hence, local production of prostaglandins (Lacroix et al., 1996; Ericsson et al., 1997; Buller et al., 1998; Lacroix and Rivest, 1998; Rivest et al., 2000). Prostaglandins in turn excite brainstem noradrenergic (A1 and A2) neurons. Activation of A2 neurons by systemic CCK is mediated via vagal afferents (Verbalis et al., 1986; Onaka et al., 1995a). Hence, systemic administration of CCK or IL-1β and forced swimming (which also utilises brainstem noradrenergic inputs to

signal to the oxytocin neurons) activate brainstem noradrenergic neurons (Luckman, 1992; Ericsson et al., 1994; Cullinan et al., 1995) to trigger noradrenaline release in the SON (Kendrick et al., 1991; Onaka et al., 1995b) and/or PVN (Brunton et al., 2005; Douglas et al., 2005) and excite magnocellular oxytocin neurons. Neurotoxin (ibotenic acid or 6-hydroxydopamine)-mediated destruction of catecholamine terminals in the SON or PVN greatly reduces activation of oxytocin neurons by systemic IL-1β (Buller et al., 2001).

Reduced responsiveness of oxytocin neurons to stress in late pregnancy

Providing uterine oxytocin receptors are upregulated (Antonijevic et al., 2000), increased oxytocin secretion during late pregnancy is likely to stimulate premature uterine contractions and increase the risk of preterm labour. In late pregnancy, the neurohypophysial oxytocin responses to some stressors are completely abolished (e.g. systemic administration of IL-1β (Fig. 1) and central administration of neuropeptide Y, NPY, an orexigenic peptide) (Brunton et al., 2006a, b), suppressed (e.g. osmotic stimulation, forced swimming) (Bull et al., 1994; Neumann et al., 1998) or unaltered (e.g. CCK) (Douglas et al., 1995). These variable responses may depend on the way these stressors signal to the magnocellular oxytocin neurons and the risk they pose in initiating preterm labour. As described above, physical stressors such as CCK, IL-1β and forced swimming converge onto NTS A2 noradrenergic neurons, as do afferents from the uterus and birth canal. SON-projecting noradrenergic NTS neurons are activated at parturition (they express Fos) (Meddle et al., 2000) and release noradrenaline in the SON (Herbison et al., 1997; Douglas et al., 2001). Different sets of NTS neurons are activated by systemic CCK and vaginal distension (Bailey and Wakerley, 1997), but the phenotypes of the NTS neurons activated by these stimuli, and hence the complement of neurotransmitters that these NTS neurons release, have not been fully defined. For instance, NTS noradrenergic neurons may co-produce NPY and enkephalins (Sawchenko et al., 1990; Matta et al., 1997). It is not known whether IL-1β (or indeed forced swimming) and uterine/birth canal afferents activate the same set of NTS neurons, although it is interesting to note that oxytocin responses to this

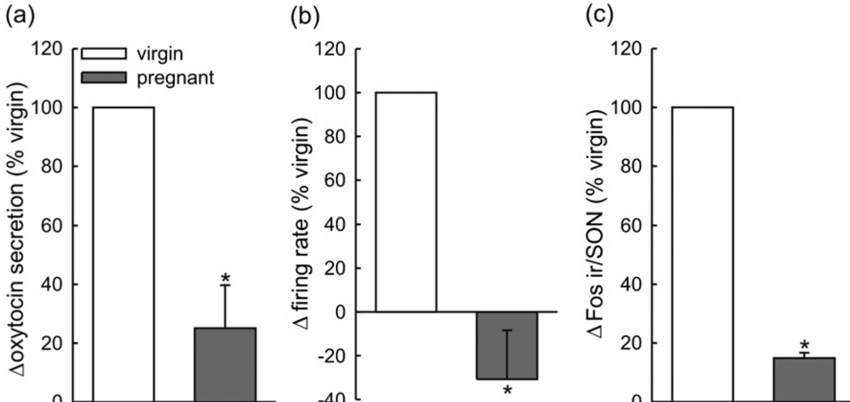

Fig. 1. Suppressed oxytocin neuron responses to systemic IL-1β in late pregnant rats. Virgin and pregnant (day 21) Sprague–Dawley rats were administered human recombinant IL-1β (500 ng/kg i.v.). (a) Mean increase in plasma oxytocin concentration in conscious rats 15 min after IL-1β administration. Group numbers: virgin, $n = 6$; pregnant, $n = 7$. (b) Mean change in the firing rate of supraoptic oxytocin neurons in urethane-anaesthetised rats 10–15 min after IL-1β administration. Group numbers: virgin, $n = 5$; pregnant, $n = 7$. (c) Mean increase in the number of neurons expressing Fos immunoreactivity in the SON 120 min after IL-1β administration in conscious rats. Group numbers: virgin, $n = 9$; pregnant, $n = 7$. In each case, data are expressed as a percentage of the mean increase in virgin rats. Error bars represent SEM. Statistics: $^{*}p < 0.001$ vs. virgin group; Student's t-test.

stressor are normally completely abolished in late pregnancy, yet infection is a major factor in preterm birth in humans (Goldenberg and Culhane, 2003) and rodents (Elovitz and Mrinalini, 2004). In late pregnancy, the persistence of oxytocin neuron excitatory responses to CCK and the suppression of responses to systemic IL-1β suggests that these stimuli activate different types of NTS neurons, producing different co-transmitters with noradrenaline.

Hyperosmotic stimuli signal directly to oxytocin neurons and via rostral circumventricular organs, rather than via brainstem inputs. Attenuated oxytocin responses to osmotic stimuli in late pregnancy result from reduced effectiveness of the excitatory rostral osmosensitive drive to oxytocin neurons. It is likely that oxytocin responses are reduced as a result of the hyponatremia of pregnancy, such that normally effective hyperosmotic stimuli are below threshold and fail to elicit a response (Bull et al., 1994).

Whatever the explanation for the suppressed oxytocin neuron responses to IL-1β in late pregnant rats, the intact excitatory electrophysiological responses of oxytocin neurons to CCK in anaesthetised late pregnant rats indicate that the neurons retain undiminished excitability. This current inference turns attention to the mechanisms that restrain the excitatory inputs to oxytocin neurons in late pregnancy.

Mechanisms of reduced oxytocin responses to stress in pregnancy

Suppressed/absent oxytocin secretory responses to stressors in late pregnancy are not attributable to reduced availability of oxytocin or a reduced capacity for secretion, since neurohypophysial stores increase by approximately 30% at the end of pregnancy (Russell et al., 2003), and blocking tonic inhibition of oxytocin neurons with picrotoxin (a gamma-aminobutyric acid A, $GABA_A$, receptor antagonist) evokes large increases in oxytocin secretion in both virgin and late pregnant rats (Russell and Brunton, 2006). Instead, suppressed responses seem to be attributable to a central mechanism that prevents/reduces activation of the oxytocin neuron cell bodies (Brunton et al., 2006b).

Endogenous opioids and noradrenergic inputs

Opioids inhibit the electrical activity of oxytocin neurons and oxytocin secretion in males and females, such that the opioid receptor antagonist, naloxone potentiates and opioid receptor agonists (e.g. morphine) inhibit stimulated oxytocin secretion (Carter et al., 1986; Brown et al., 2000). Opioids can exert their inhibitory effect by acting directly on oxytocin neurons in the SON and PVN, or presynaptically on afferent inputs (Brown et al., 2000). Oxytocin neuron cell bodies express both μ- and κ-opioid receptors (Sumner et al., 1992), and enkephalins and dynorphins are co-expressed in oxytocin neurons in the SON and PVN and are co-released with oxytocin from nerve terminals in the posterior pituitary where they auto-regulate oxytocin secretion (Meister et al., 1990).

In early-to-mid pregnancy, endogenous opioids act on κ-receptors to preterminally inhibit oxytocin secretion at the neural lobe. However, opioid inhibition at the posterior pituitary declines in late pregnancy and instead oxytocin neurons are inhibited by a central μ-opioid receptor-mediated mechanism. This central opioid restraint emerges in late pregnancy (after day 16) (Douglas et al., 1995) and remains effective through parturition, where it plays an important role in regulating oxytocin secretion, and hence optimising interbirth intervals (Leng et al., 1988). This central opioid inhibition is revealed by the effects of administering naloxone, which greatly augments oxytocin secretion after stimulation by forced swimming (Douglas et al., 1998), intravenous (i.v.) CCK (Douglas et al., 1995) or IL-1β (Brunton et al., 2005) and enhances the firing of supraoptic oxytocin neurons evoked by CCK (Douglas et al., 1995) and IL-1β (Brunton et al., 2006b) in late pregnancy (Fig. 2).

Expression of $\alpha 1_A$-adrenoceptor mRNA expression is reduced in the magnocellular region of the PVN in late pregnancy (Douglas et al., 2005), which may contribute to reduced activation of the oxytocin neurons by stimuli utilising noradrenergic inputs. However, perhaps more importantly,

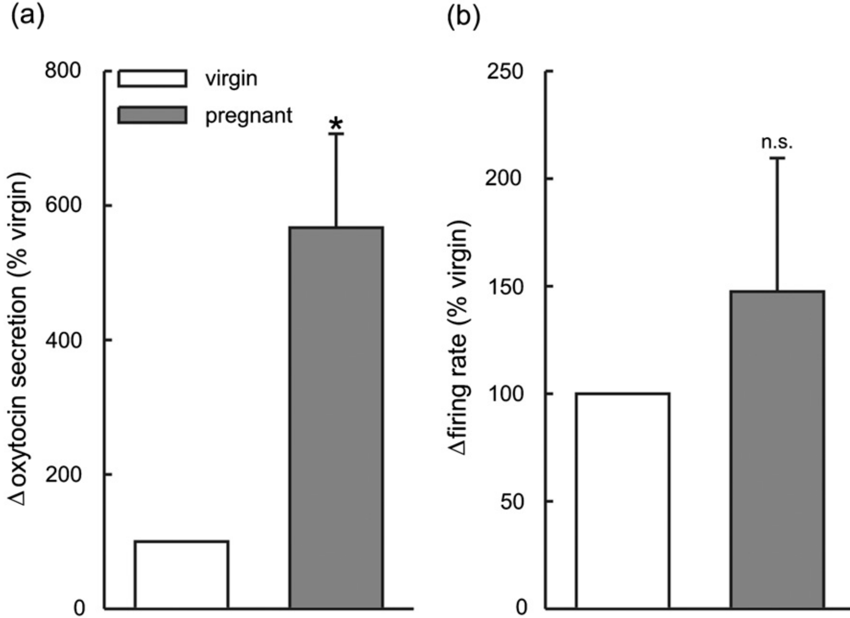

Fig. 2. Unrestrained oxytocin neuron responses to systemic IL-1β after naloxone treatment in late pregnant rats. Virgin and pregnant (day 21) Sprague–Dawley rats were administered naloxone (5 mg/kg i.v.) 15 min before IL-1β (500 ng/kg i.v.) administration. (a) Mean increase in plasma oxytocin concentration in conscious rats 15 min after IL-1β administration. Group numbers: virgin, $n = 5$; pregnant, $n = 7$. (b) Mean increase in the firing rate of supraoptic oxytocin neurons in urethane-anaesthetised rats 10–15 min after IL-1β administration. Group numbers: virgin, $n = 5$; pregnant, $n = 5$. In both cases, data are expressed as a percentage of the mean increase in virgin rats. Error bars represent SEM. Statistics: $^{*}p < 0.01$; Student's *t*-test; n.s., not significant.

in late pregnancy, both systemic IL-1β (Brunton et al., 2005) and forced swimming (Douglas et al., 2005) fail to evoke noradrenaline release in the PVN, despite the apparent unaltered signalling to the NTS neurons (Brunton et al., 2005); thus explaining the lack of response by the oxytocin neurons to these stressors. IL-1β-induced noradrenaline release in the PVN is inhibited by endogenous opioids in late pregnancy and can be reinstated by local infusion of naloxone (Brunton et al., 2005), providing further support for the proposal that in late pregnancy opioids presynaptically inhibit noradrenergic inputs to the oxytocin neurons.

There are several potential sources of the opioids involved in centrally inhibiting oxytocin neuron responses to stressors at the end of pregnancy. Proenkephalin-A is expressed in the PVN and perifornical region and dynorphin is expressed in the SON; however, mRNA expression for these opioids in these regions is unaltered in pregnancy (Douglas et al., 1993; Brunton et al., 2005). Opioids are expressed in several brain regions that innervate magnocellular oxytocin neurons. Arcuate nucleus neurons project to the PVN and SON and β-endorphin (a product of the pro-opiomelanocortin, POMC, gene) is expressed in the arcuate nucleus (Sawchenko et al., 1982). Moreover, POMC and β-endorphin expression is enhanced in the arcuate nucleus, and the density of β-endorphin immunoreactive nerve fibres in and around the SON increases in pregnancy (Douglas et al., 2002). Nevertheless, arcuate neurons are unlikely to mediate the rapid actions of opioids on oxytocin neurons following stress, since activation of arcuate neurons by stressors (e.g. IL-1β) lags somewhat behind those involved in processing stress stimuli (Brady et al., 1994), and oxytocin neuron responses are more rapid. The NTS is the most likely source of opioids that inhibit oxytocin neuron responses to stressors in pregnancy. NTS neurons synthesise enkephalins and μ-opioid

receptors; the mRNA expression for both proenkephalin and μ-opioid receptor mRNA is up-regulated in late pregnant rats (Brunton et al., 2005). It is not known whether the NTS neurons expressing up-regulated opioid in pregnancy are the same neurons that are activated by IL-1β. If, however, this is the case then it would provide a mechanism by which opioids could presynaptically inhibit noradrenaline release in the PVN and SON in response to IL-1β (and any other stressors that activate this set of NTS neurons), hence preventing activation of the oxytocin neurons and subsequent oxytocin secretion.

Sex steroids and endogenous opioids

Plasma concentrations of the female sex steroids, oestrogen and progesterone increase during pregnancy in the rat, making them putative candidates in signalling the pregnancy status of the animal to the brain and hence evoking adaptations in neuroendocrine responses to stress. Oestradiol secretion increases steadily during gestation, reaching a peak just before term, whereas progesterone secretion increases rapidly after mating, plateaus at early-mid gestation (between days 7 and 10), increases and plateaus again during the last week of pregnancy (between days 15 and 20) before collapsing prior to parturition (Mann and Bridges, 2001).

A distinct sexual dimorphism exists in opioid regulation of stress-induced oxytocin secretion from the posterior pituitary which is, at least in part, attributable to circulating gonadal hormones. The oxytocin secretory response to immobilisation is potentiated by naloxone treatment in female, but not male rats, unless the gonadal influence is removed by prior castration (Carter et al., 1986). Mimicking hormone changes of pregnancy in virgin rats with chronic oestradiol and progesterone treatment results in a modest reduction in the oxytocin secretory response to forced swimming, similar to that seen in late pregnancy (Douglas et al., 1998). However, when the actions of endogenous opioids are blocked with naloxone, the oxytocin response is greatly enhanced (Douglas et al., 2000). Thus, opioid inhibition of oxytocin responses to stress can be induced in virgin rats by treatment with pregnancy levels of oestradiol and progesterone, although relaxin may also play an important role in pregnancy (Way et al., 1993). In contrast, combined oestrogen and progesterone treatment does not alter the ACTH and corticosterone responses to stress, even in the presence of naloxone (Douglas et al., 2000), indicating that these sex steroids are neither responsible for attenuated HPA axis responses to stress observed in pregnancy nor involved in induction of opioid regulation of the HPA axis.

Sex steroids can undoubtedly modulate the expression of opioids in the hypothalamus: the promoter region of the enkephalin gene contains an oestrogen response element (Zhu and Pfaff, 1995), and oestradiol up-regulates proenkephalin gene transcription in the ventromedial nucleus (Romano et al., 1989). Earlier we proposed that up-regulated opioid in NTS neurons was of primary importance in restraining oxytocin neuron responses, at least in response to stressors that signal through brainstem noradrenergic neurons. However, it is unlikely that the female sex steroids per se act on the NTS neurons to increase the expression of opioids in pregnancy, as SON-projecting NTS A2 neurons do not express oestrogen receptors (Voisin et al., 1997) and are almost devoid of progesterone receptors (Francis et al., 2002).

Allopregnanolone and endogenous opioids

In pregnancy, the concentrations of allopregnanolone, a neuroactive metabolite of progesterone, increase in both plasma and the brain, peaking around day 19 in the rat (Concas et al., 1998). Progesterone is converted into 5α-dihydroprogesterone and then allopregnanolone (5α-pregnan-3α-ol-20-one; 3α,5α-tetrahydroprogesterone) by the actions of 5α-reductase and 3α-hydroxysteroid dehydrogenase, respectively, which are both expressed centrally (Gao et al., 2002). Allopregnanolone acts in a barbiturate-like manner to modulate central $GABA_A$ receptors, enhancing their function (Majewska, 1992). There are copious GABA synapses on oxytocin neurons, and in late pregnancy the GABA input is more effective due to an increased number of GABA synapses (Koksma et al., 2005;

Theodosis et al., 2006) and the actions of allopregnanolone (Brussaard et al., 1999).

Oxytocin secretory responses to IL-1β can be reinstated in late pregnant rats by blocking allopregnanolone production with the 5α-reductase inhibitor, finasteride (Russell and Brunton, 2006). In addition, oxytocin secretion following IL-1β can be suppressed in non-pregnant rats, by prior treatment with allopregnanolone (Russell and Brunton, 2006) (Fig. 3). Evidently, these effects are mediated centrally as finasteride increases, while allopregnanolone decreases IL-1β-induced Fos expression in identified magnocellular oxytocin neurons in the PVN and SON in late pregnant and virgin rats, respectively (Brunton et al., unpublished). Whether allopregnanolone also restrains oxytocin neuron responses to other stressors in late pregnancy (e.g. forced swimming) remains to be elucidated. Moreover, it is unclear how allopregnanolone may exert these effects, and what involvement $GABA_A$ receptors on oxytocin or NTS neurons may have. However, it is likely that endogenous opioids are involved in mediating allopregnanolone actions.

Central opioid inhibition of oxytocin secretion is attributable, at least in part, to the actions of sex steroids and relaxin (see above); nevertheless, short-term allopregnanolone treatment also induces inhibitory opioid tone over oxytocin responses to IL-1β in virgin rats (Brunton et al., unpublished). Furthermore, allopregnanolone treatment up-regulates the expression of mRNA for proenkephalin-A in NTS neurons (which provide afferent inputs to the SON and PVN) by an extent akin to that seen in late pregnant rats (Brunton et al., 2005). The mechanism underpinning the regulation of opioid expression by allopregnanolone has yet to be determined.

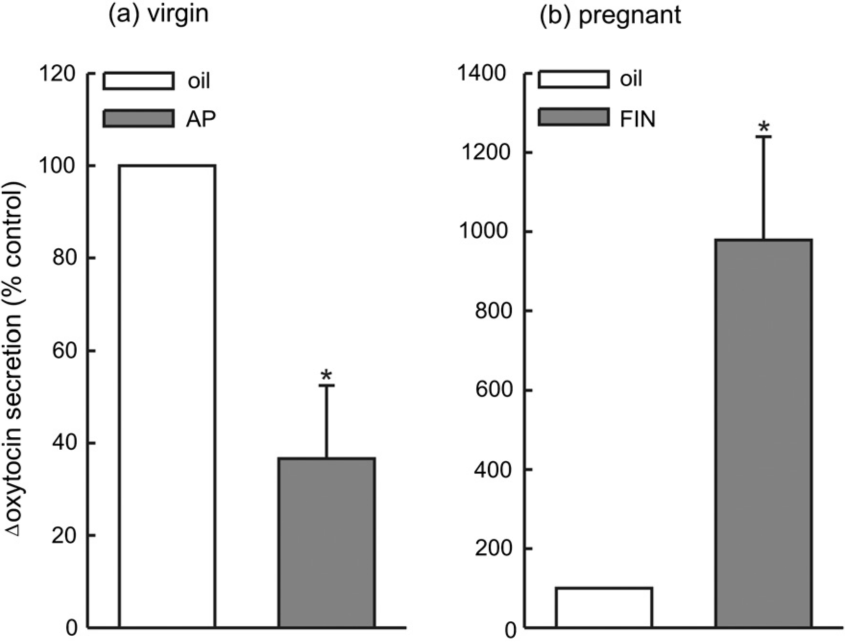

Fig. 3. Allopregnanolone control of oxytocin secretory responses to IL-1β. (a) Mean increase in plasma oxytocin concentration 15 min after IL-1β (500 ng/kg i.v.) administration in virgin rats pretreated with either vehicle (corn oil) or allopregnanolone (3 mg/kg and 1 mg/kg s.c., respectively) 20 and 2 h before IL-1β. Group numbers: virgin, $n = 7$; pregnant, $n = 6$. (b) Mean increase in plasma oxytocin concentration 15 min after IL-1β (500 ng/kg i.v.) administration in pregnant (day 21) rats pretreated with either vehicle (sesame oil) or finasteride (5α-reductase inhibitor, blocks allopregnanolone production; 25 mg/kg s.c.) 20 and 2 h before IL-1β. Group numbers: virgin, $n = 7$; pregnant, $n = 6$. In both cases, data are expressed as a percentage of the mean increase in the control group. Error bars represent SEM. Statistics: $^*p < 0.004$; Student's t-test.

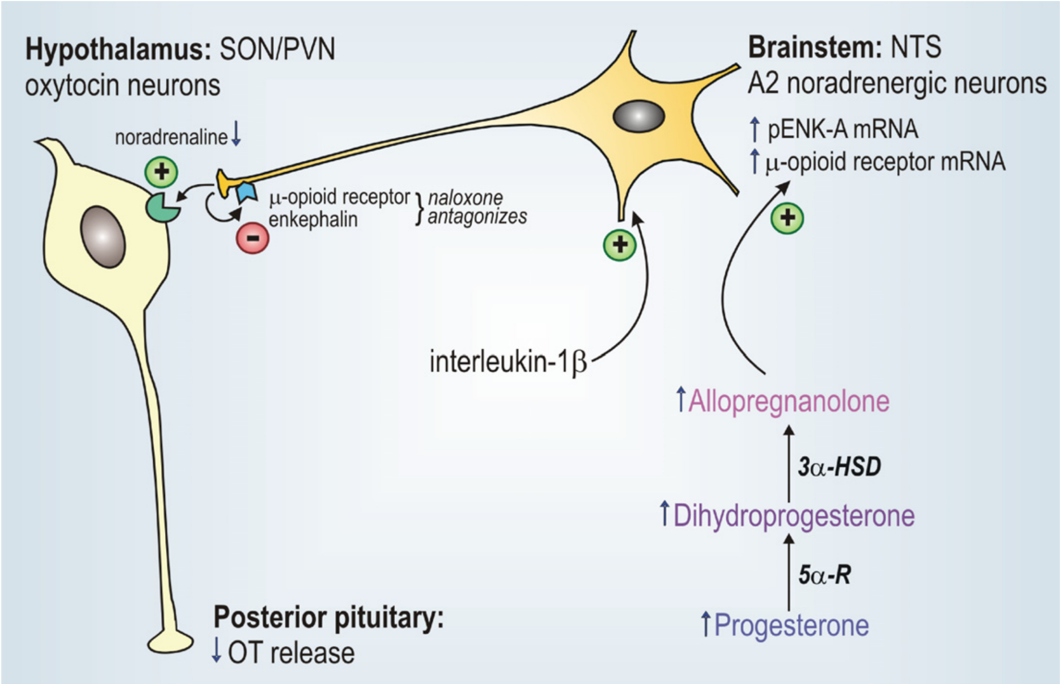

Fig. 4. Mechanisms of reduced oxytocin neuron responses to interleukin-1β (IL-1β) in late pregnancy. Circulating and brain progesterone levels are increased (↑) in pregnancy. Progesterone is converted into dihydroprogesterone and allopregnanolone by 5α-reductase (5α-R) and 3α-hydroxysteroid dehydrogenase (3α-HSD), respectively. Allopregnanolone up-regulates proenkephalin-A (pENK-A) mRNA and possibly μ-opioid receptor mRNA expression in nucleus tractus solitarii (NTS) neurons. Noradrenergic A2 neurons project to the supraoptic (SON) and paraventricular nuclei (PVN) and innervate magnocellular oxytocin neurons. Systemic (IL-1β) activates (+) these brainstem neurons; however, in pregnancy, IL-1β fails to evoke noradrenaline release from their terminals in the PVN. This is a consequence of increased opioid inhibition (by enkephalin) acting presynaptically on μ-opioid receptors on the noradrenergic nerve terminals. Naloxone (an opioid receptor antagonist) given systemically restores oxytocin neuron firing and secretory responses to IL-1β, or infused directly into the PVN can restore IL-1β-evoked noradrenaline release. This inhibitory (–) opioid mechanism, induced by the increased levels of allopregnanolone in pregnancy, prevents activation of the oxytocin neurons and results in reduced oxytocin secretion (↓). (See Color Plate 30.4 in color plate section.)

Summary

In late pregnancy, oxytocin neurons are prevented from premature activation by stressors such as IL-1β (Fig. 4). The restraining mechanism relies on presynaptic inhibition of excitatory noradrenergic inputs to the oxytocin neurons by endogenous opioids, rather than on changes in excitability of the oxytocin neurons. The progesterone metabolite, allopregnanolone, plays an important in role in inducing this inhibitory opioid regulation of oxytocin neuron activity in late pregnancy. These adaptations will prevent depletion of the expanded oxytocin stores in the posterior pituitary which are required at parturition and minimise the risk of preterm labour.

Abbreviations

ACTH	adrenocorticotrophic hormone
CCK	cholecystokinin
HPA	hypothalamo-pituitary-adrenal
GABA	gamma-aminobutyric acid
IL-1β	interleukin-1 beta
NPY	neuropeptide Y
NTS	nucleus tractus solitarii
POMC	pro-opiomelanocortin

PVN paraventricular nucleus
SON supraoptic nucleus
VLM ventrolateral medulla

Acknowledgements

The authors would like to thank Juliana Bales, Helen Cameron and Ailsa MacKay for their contribution to some of the work presented here. Financial support was provided by the Biotechnology and Biological Sciences Research Council (BBSRC).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 31

Rapid synapse-specific regulation of hypothalamic magnocellular neurons by glucocorticoids

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Abstract: Glucocorticoids secreted in response to stress activation of the hypothalamic–pituitary–adrenal axis feed back onto the hypothalamus to rapidly suppress neuroendocrine activation, including oxytocin and vasopressin secretion. Here we provide a brief review focused on our recent findings of a rapid glucocorticoid-induced opposing regulation of glutamate and γ-aminobutyric acid (GABA) inputs to magnocellular neurons via the release of distinct retrograde messengers. The stress hormone corticosterone and its synthetic analogue dexamethasone elicit the rapid retrograde release of endocannabinoids by activating a novel membrane-associated, G protein-coupled receptor in parvocellular and magnocellular neuroendocrine cells of the hypothalamic paraventricular and supraoptic nuclei. Glucocorticoids also cause the rapid retrograde release of an unknown messenger that facilitates presynaptic GABA release onto magnocellular neuroendocrine cells. These finding suggest that there is a strict synapse-specific segregation of the opposing actions of the two retrogradely released messengers. Thus, the combined actions of glucocorticoids cause a rapid synaptic inhibition of the magnocellular neurons and would be expected, therefore, to mediate a rapid feedback inhibition of the secretion of oxytocin and vasopressin during stress activation of the hypothalamic–pituitary–adrenal axis.

Keywords: magnocellular; SON; PVN; vasopressin; oxytocin; mEPSC; mIPSC; GABA; glutamate; glucocorticoid; retrograde messenger

Glucocorticoids released from the adrenal cortex in response to stress activation of the hypothalamic–pituitary–adrenal (HPA) axis have an extensive range of effects on physiological and behavioural responses. Among the actions of circulating glucocorticoids are negative feedback effects on the brain and pituitary gland. A main feedback effect of glucocorticoids is to suppress the activation of the HPA axis, inhibiting HPA hormone secretion and terminating the neuroendocrine stress response. These inhibitory feedback effects on HPA axis activation are thought to occur in the hippocampus, hypothalamus and pituitary gland (Keller-Wood and Dallman, 1984; Dallman et al., 1987; Saphier and Feldman, 1988; Young et al., 1990). The glucocorticoid feedback regulation occurs both acutely, by a rapid inhibition of corticotropin releasing hormone (CRH) release, and more slowly, by a protracted down-regulation of CRH and vasopressin expression in CRH neurons of the paraventricular nucleus

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DOI: 10.1016/S0079-6123(08)00431-7

(PVN) (Keller-Wood and Dallman, 1984; Young et al., 1990). While the slow feedback effect is mediated by well-characterized genomic actions of the steroid at the classical intracellular glucocorticoid receptors, the rapid effects are inconsistent with a transcriptional action of the steroid and invoke a mechanism involving a membrane glucocorticoid receptor.

Evidence for nongenomic glucocorticoid actions mediated by putative membrane receptors has accumulated over the last 30 years from studies in different species and different cell types, although a specific membrane glucocorticoid receptor(s) has not been identified to date. Studies in the salamander, however, have isolated a putative membrane receptor responsible for the rapid corticosteroid inhibition of male reproductive behaviour that has binding properties characteristic of a G protein-coupled receptor (Orchinik et al., 1991, 1992; Evans et al., 2000). The rapid glucocorticoid actions in the newt activated by this putative membrane glucocorticoid receptor are thought to be mediated by the release of endocannabinoids (Coddington et al., 2007), which is a similar rapid glucocorticoid action to that which we have characterized in the rat hypothalamus (see below). Additionally, several labs have reported rapid effects of glucocorticoids on membrane electrical properties of neurons (Chen et al., 1991; ffrench-Mullen, 1995; Chen and Qiu, 2001) that suggest activation of one or more membrane receptors and downstream activation of protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and mitogen-activated protein (MAP) kinase signalling pathways (Qiu et al., 1998, 2003; Han et al., 2005; Qi et al., 2005).

Glucocorticoid effects on central neuroendocrine function impact more than just the HPA axis, as glucocorticoids have been shown to exert inhibitory actions in different neuroendocrine systems (Sapolsky et al., 1990; Papanek et al., 1997; Di et al., 2003), including in the oxytocin- and vasopressin-secreting magnocellular neuroendocrine cells of the hypothalamic PVN and supraoptic nucleus (SON). Here we present a brief review that focuses on recent studies on rapid glucocorticoid actions in SON and PVN magnocellular neurons.

Rapid glucocorticoid feedback regulation in the hypothalamus

Glucocorticoids have been found to exert effects on neuronal activity that occur within seconds to minutes of the exposure of the cells to the steroid, precluding the involvement of genomic regulation in mediating these effects. The transcription-independent glucocorticoid actions in the main glucocorticoid feedback target sites tend predominantly toward the rapid suppression of activation of the HPA axis, as would be predicted for a negative feedback regulation. Thus, at the pituitary, glucocorticoids suppress CRH-induced ACTH secretion within minutes via a rapid, transcription-independent mechanism (Widmaier and Dallman, 1984; Hinz and Hirschelmann, 2000). The rapid glucocorticoid effect on the pituitary appears to account for about half of the total rapid feedback inhibition of ACTH release in vivo (Jones et al., 1977; Keller-Wood and Dallman, 1984), the remaining half occurring within the brain.

Glucocorticoids have been reported to exert primarily inhibitory rapid effects on the spiking activity of median eminence-projecting PVN neurons recorded in vivo (Kasai and Yamashita, 1988b; Saphier and Feldman, 1988; Chen et al., 1991). This inhibitory effect on spiking activity has also been observed in PVN neurons recorded in slices of hypothalamus in vitro (Chen et al., 1991), although these effects were blocked by the intracellular glucocorticoid receptor antagonist, RU486. In another series of studies in hypothalamic slices, cortisol generally did not affect the spontaneous spiking frequency of PVN neurons recorded in the parvocellular subdivision (Kasai and Yamashita, 1988a), although it blocked spike activation induced in these neurons by norepinephrine, suggesting an indirect effect on the noradrenergic modulation of synaptic inputs. It should be noted that glucocorticoids also have been reported to suppress voltage-activated potassium currents (Zaki and Barrett-Jolley, 2002) and to enhance the spiking activity of some PVN neurons (Chen et al., 1991; Zaki and Barrett-Jolley, 2002), suggesting a possible excitatory roll in some cells of the PVN.

Glucocorticoids have also been reported to have rapid inhibitory effects on oxytocin and

vasopressin secretion from hypothalamic magnocellular neurons. Corticosterone inhibits osmotically-stimulated vasopressin secretion from hypothalamic explants that include the SON, but not the PVN, suggesting an effect specifically on vasopressin release from magnocellular neurons (Liu et al., 1995; Papanek et al., 1997). This effect appears to be mediated by activation of a membrane corticosteroid receptor because it is also obtained in slices with application of corticosterone conjugated to bovine serum albumin, which does not cross the membrane (Liu and Chen, 1995). The inhibitory effect of corticosteroids on magnocellular neuron vasopressin release has been linked to the glutamatergic excitatory synaptic inputs activated by osmotic stimulation of the organum vasculosum (Sladek et al., 2000).

Mechanisms of rapid glucocorticoid regulation of magnocellular neurons

The reported rapid inhibitory effects of glucocorticoids on hypothalamic SON and PVN magnocellular neurons suggest a suppression of excitatory synaptic drive and/or a facilitation of inhibitory synaptic input to these neurons. This is supported by our recent studies, which have focused on the rapid modulation by glucocorticoids of excitatory and inhibitory synaptic inputs to multiple subtypes of neuroendocrine cells of the hypothalamic PVN and SON using whole cell patch clamp recordings in an in vitro rat brain slice preparation.

Rapid glucocorticoid suppression of glutamate release

While without effect on postsynaptic properties such as membrane holding current and membrane resistance, glucocorticoids were seen to cause a rapid suppression of glutamate-dependent excitatory synaptic inputs to SON and PVN magnocellular neurons (Fig. 1). Dexamethasone (DEX), a synthetic glucocorticoid, and corticosterone, the endogenous glucocorticoid found in rodents, both elicited a dose-dependent decrease in the frequency of glutamatergic miniature excitatory postsynaptic currents (mEPSCs), with a half-maximal glucocorticoid response at around 500 nM (Di et al., 2003, 2005b). The glucocorticoid effect was rapid in onset, occurring within 1–3 min, and was mediated by the activation of a membrane receptor, as it was maintained when DEX was restricted to the extracellular compartment by conjugation to membrane-impermeant bovine serum albumin and it was abolished by the direct intracellular application of unconjugated DEX via the patch pipette. This indicated a non-classical mechanism of action of the steroid by activation of a novel, membrane-associated receptor. The effect was inhibited by blocking G protein activity in the cells, suggesting that it may be mediated by the activation of a G protein-coupled receptor in the neuroendocrine cells. Since a modulation of neurotransmitter release indicates a presynaptic mechanism of action, the blockade of the glucocorticoid response by inhibition of postsynaptic G protein activity suggested the involvement of a retrograde signal from the postsynaptic cell to the presynaptic glutamate terminals. Consistent with this, glucocorticoids were found to elicit the synthesis of the endocannabinoids (eCBs) arachidonoylethanolamine (AEA, anandamide) and 2-arachidonoylglycerol (2-AG) (Di et al., 2005a; Malcher-Lopes et al., 2006). Endocannabinoids recently have been identified as a major class of retrograde messengers at excitatory and inhibitory synapses, where they are synthesized on demand from lipid precursors in the postsynaptic membrane and ''released'' in a retrograde fashion to act on cannabinoid CB1 receptors at glutamate and GABA terminals to inhibit transmitter release (Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001; Alger, 2002). We and others have found that CB1 receptors are expressed at glutamate and GABA terminals in the SON and PVN, and that activation of these receptors by endogenous as well as exogenously applied cannabinoids suppresses glutamate and GABA release onto the magnocellular neurons (Hirasawa et al., 2004; Di et al., 2005b; Soya et al., 2005). Consistent with endocannabinoids serving as retrograde messengers in the inhibitory effect of glucocorticoids on glutamate inputs, the glucocorticoid suppression of glutamate synaptic inputs to the magnocellular neurons was blocked by CB1 cannabinoid receptor antagonists and it was mimicked and occluded by

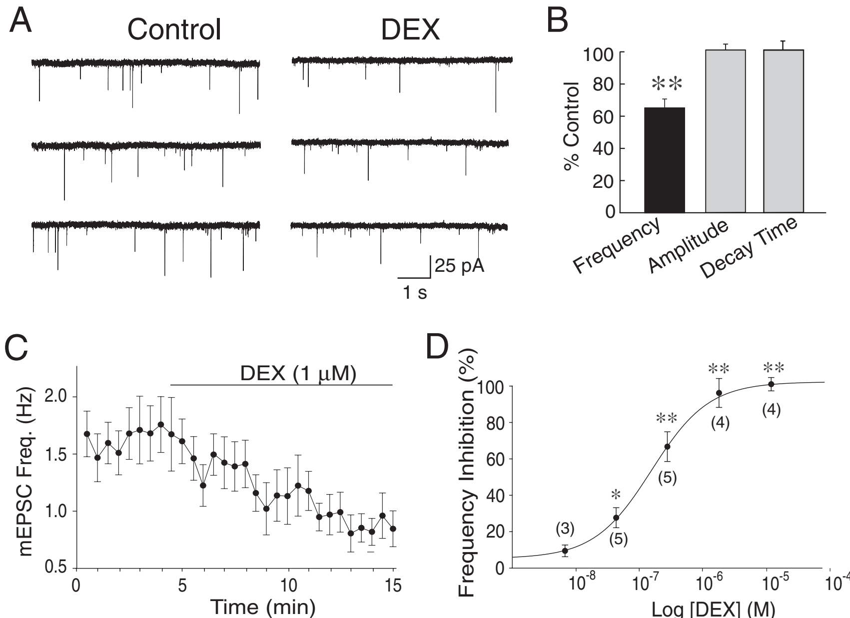

Fig. 1. Glucocorticoid-induced rapid, dose-dependent suppression of glutamate release onto magnocellular neurons of the SON. (A) Whole-cell recording of mEPSCs in normal medium (Control) and after 7 min in medium containing dexamethasone (DEX, 1 μM). (B) DEX caused a significant reduction in the mEPSC frequency, but not amplitude or decay time. (C) Time course analysis of DEX effect showed mEPSC frequency decreased within 3 min of DEX application (mean ± SE, 30 s bins). (D) Dose-dependence of the DEX-induced decrease in mEPSC frequency. Percent reduction in mEPSC frequency is plotted against increasing concentrations of DEX and curve fitted. $EC_{50} = 474$ nM. *, $p<0.05$; **, $p<0.01$. Adapted with permission from Di et al., 2005b.

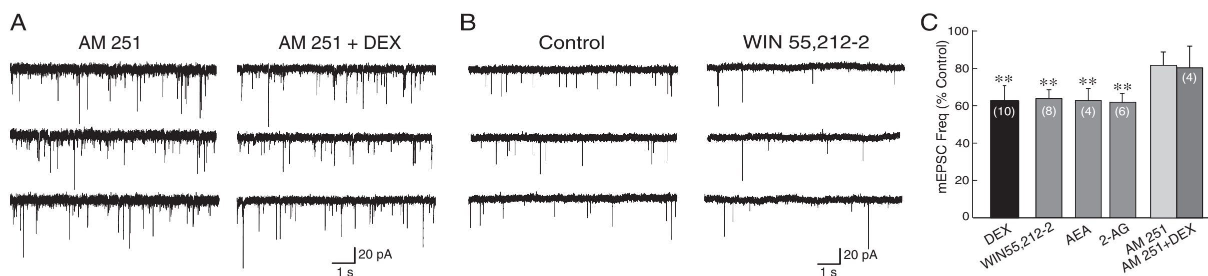

Fig. 2. Glucocorticoid-induced suppression of glutamate release is mediated by a retrograde endocannabinoid messenger. (A) The CB1 receptor antagonist, AM251 (1 μM), blocked DEX-induced inhibition of mEPSCs. (B) The synthetic CB1 receptor agonist, WIN 55,212-2, mimicked the glucocorticoid effect, causing a selective reduction of mEPSC frequency. (C) Average change in mean mEPSC frequency showed that the synthetic cannabinoid (WIN 55,2212-2), and endocannabinoids (AEA, 2-AG) mimicked the DEX effect and the CB1 antagonist (AM251) blocked the DEX effect. **, $p<0.01$. Adapted with permission from Di et al., 2005b.

exogenously applied CB1 agonists (Fig. 2) (Di et al., 2003, 2005b). This suggested, therefore, that glucocorticoids rapidly suppress excitatory synaptic transmission in magnocellular neurons by inducing the postsynaptic synthesis and retrograde release of endocannabinoids, which inhibit the release of glutamate from excitatory afferents via activation of presynaptic CB1 receptors.

We determined the intracellular signalling pathway involved in endocannabinoid synthesis by the

putative membrane glucocorticoid receptor by studying the interaction of the receptor with the leptin receptor. The adipocyte peptide hormone leptin serves as a satiety signal to the brain by activation of a receptor tyrosine kinase and its rapid actions are mediated, in part, by downstream actions on a PI3 kinase signalling pathway (Sahu, 2003). Leptin has been shown to block fasting-induced endocannabinoid synthesis in the hypothalamus, which may contribute to its effect on satiety (Di Marzo et al., 2001). We found that leptin blocks the rapid glucocorticoid-induced endocannabinoid synthesis and endocannabinoid-mediated suppression of glutamate release in both magnocellular and parvocellular neurons of the PVN (Malcher-Lopes et al., 2006), and that this effect was mediated by activation of phospodiesterase 3B (PDE-3B). Since PDE-3B acts by converting cAMP to its inactive 5′AMP form, this indicated that the putative membrane glucocorticoid receptor was acting through a cAMP pathway to stimulate endocannabinoid biosynthesis. Blocking the α subunit of the stimulatory G protein (Gs) as well as cAMP and cAMP-dependent protein kinase (PKA) inhibited the rapid glucocorticoid effect on endocannabinoid synthesis and glutamate release (Malcher-Lopes et al., 2006), strongly implicating this pathway in the rapid actions of glucocorticoids on endocannabinoid release. These findings also suggested that the rapid glucocorticoid actions are mediated by the activation of a putative membrane G protein-coupled receptor.

Rapid glucocorticoid facilitation of GABA release

In addition to the suppression of excitatory synaptic transmission, we also found that glucocorticoids cause the rapid facilitation of the release of synaptic GABA onto magnocellular neurons (Fig. 3) (Di et al., 2005b). The rapid glucocorticoid facilitation of GABA release is also mediated by

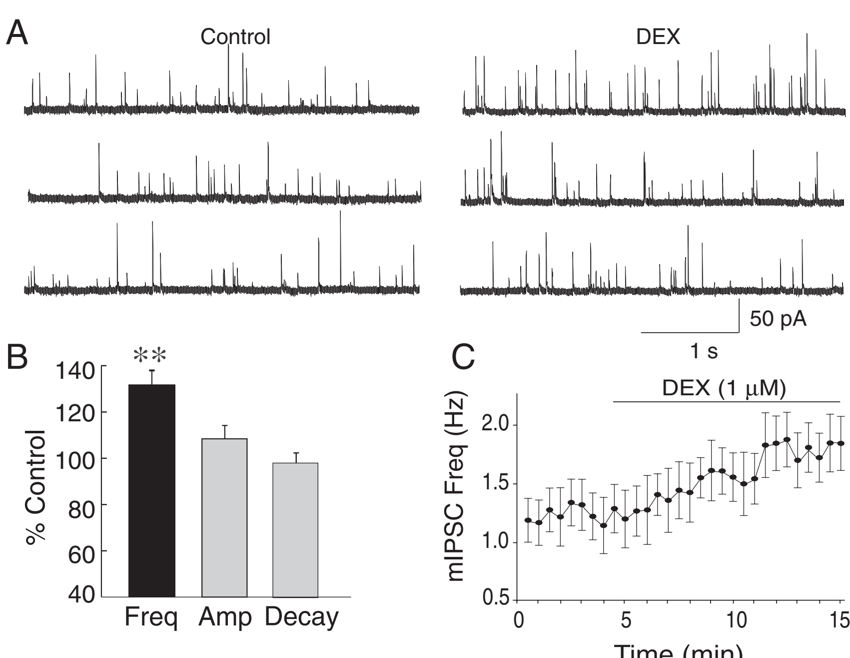

Fig. 3. Glucocorticoid-induced facilitation of GABA release onto magnocellular neurons. (A) Whole-cell recording of mIPSCs in normal medium (Control) and after 7 min in medium containing DEX (1 μM). (B) DEX caused a significant increase in the mIPSC frequency, with no change in the amplitude or decay time. (C) Time course of DEX effect showed mIPSC frequency increased within 3 min of DEX application (mean ± SE, 30 s bins). **, $p < 0.01$. Adapted with permission from Di et al., 2005b.

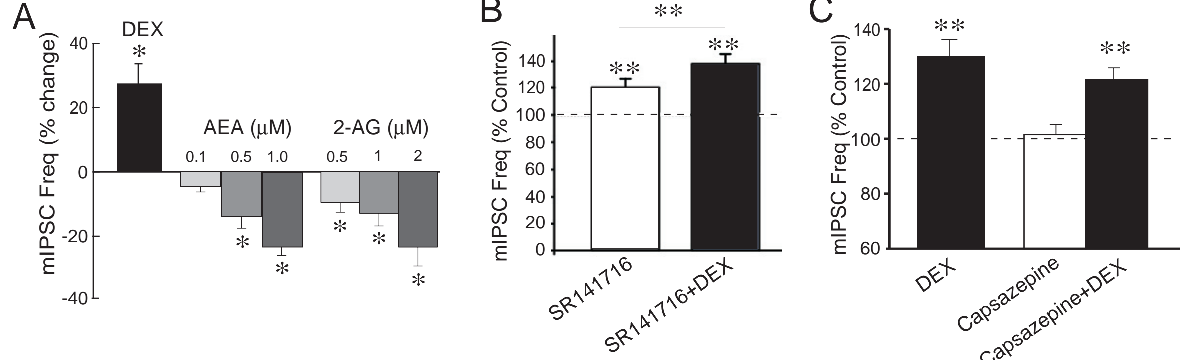

Fig. 4. Glucocorticoid-induced facilitation of GABA release is mediated by an unknown retrograde messenger. (A) DEX (1 μM) caused a significant increase in mIPSC frequency, whereas the endocannabinoids AEA and 2-AG elicited the opposite effect, a dose-dependent decrease in mIPSC frequency. (B) The DEX-induced increase in mIPSC frequency was not blocked by the CB1 receptor antagonist SR 141716 (1 μM). Note SR 141716 increased basal mIPSC frequency. (C) The DEX-induced increase in mIPSC frequency was not blocked by TRPV1 receptor antagonist capsazapine (10 μM). **, $p < 0.01$.

the activation of a postsynaptic membrane-associated receptor, is dependent on postsynaptic G protein activity, and involves the release of a retrograde messenger, although it is unlikely that the retrograde messenger is an endocannabinoid that acts at CB1 receptors. Thus, unlike glucocorticoids, exogenously applied cannabinoids do not facilitate, but rather suppress, GABA release (Di et al., 2005b), and the glucocorticoid-induced facilitation of GABA release is not blocked by the CB1 receptor antagonist SR141716 (Fig. 4). This is in contrast to previous findings showing that the facilitatory effect on GABA release was blocked by another CB1 antagonist, AM251 (Di et al., 2005b), suggesting that AM251, therefore, exhibits non-specific effects. The endocannabinoid AEA has been shown to serve as an endogenous ligand at transient receptor potential vanilloid 1 (TRPV1) receptors, where it causes a facilitation of transmitter release (Di Marzo et al., 2002). We found that the facilitatory effect of glucocorticoids was not prevented by the TRPV1 antagonist capsazepine, suggesting that it was not caused by endocannabinoids acting at TRPV1 receptors on GABA terminals (Fig. 4). Finally, in contrast to the glucocorticoid suppression of glutamate release (Malcher-Lopes et al., 2006), the rapid glucocorticoid facilitation of GABA release was not blocked by the adipocyte hormone leptin (Di and Tasker, unpublished observation). Therefore, the rapid glucocorticoid facilitation of inhibitory synaptic input to the magnocellular neurons appears to be mediated by a distinct retrograde messenger. Experiments are currently underway to identify this second retrograde messenger. A potential candidate is nitric oxide (NO), which is an important biological signal that plays a critical role in a variety of physiological processes. It has been observed that glucocorticoids induce endothelial NO release by stimulating nitric oxide synthesis, thereby contributing to the acute cardiovascular protective effect of the steroid (Hafezi-Moghadam et al., 2002). Magnocellular neurons of the PVN and SON show robust NO synthase expression (Yuan et al., 2006), and application of NO precursors facilitates GABA release onto PVN and SON magnocellular and parvocellular neurons in vivo (Stern and Ludwig, 2001; Li et al., 2002).

Conclusion

Our recent work has suggested, as illustrated in our proposed working model (Fig. 5), that glucocorticoids activate a membrane-associated, G protein-coupled receptor to stimulate the synthesis and retrograde release of the endocannabinoids

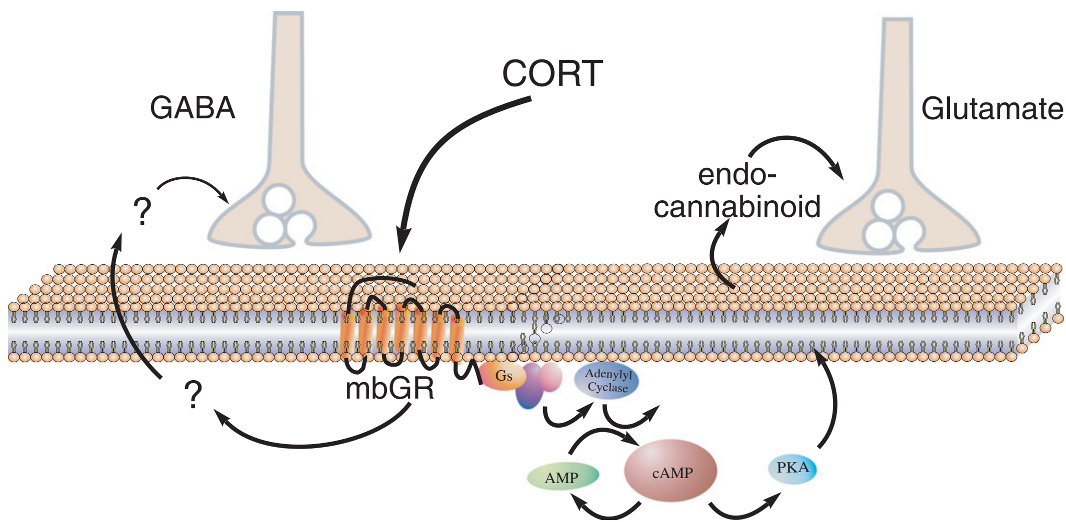

Fig. 5. Proposed model of rapid glucocorticoid effects on excitatory and inhibitory synaptic inputs to neuroendocrine cells of the PVN and SON. Glucocorticoid (GC) activation of a membrane GC receptor (mbGR) in magnocellular neuroendocrine cells activates a G protein-coupled signalling pathway that results in the synthesis and release of endocannabinoids (CB). The endocannabinoids are transmitted retrogradely to presynaptic glutamate terminals where they suppress glutamate release (-) via actions at CB1 receptors. GCs also cause a rapid facilitation of GABA-mediated inhibitory synaptic inputs to magnocellular neurons via the release of an unknown retrograde messenger. (See Color Plate 31.5 in color plate section.)

AEA and 2-AG from magnocellular neuroendocrine cells, which leads to the suppression of excitatory synaptic inputs. In addition, glucocorticoids elicit a rapid facilitation of GABA release onto magnocellular neurons, which is also mediated by the activation of a putative membrane G protein-coupled receptor, presumably the same receptor, and the synthesis and release of a retrograde messenger, albeit a different messenger. These rapid effects of glucocorticoids were seen in both oxytocin and vasopressin neurons (Di et al., 2005b).

Of particular interest is the finding that glucocorticoids cause the release of apparently different retrograde messengers at neighbouring glutamate and GABA synapses, and these have opposite effects at the two types of synapses, suppressing glutamate release and facilitating GABA release. Glutamate and GABA synapses on magnocellular neurons are located in close physical proximity to one another (Theodosis et al., 1995). The glucocorticoid-induced, retrogradely released endocannabinoids cause a suppression of synaptic glutamate release, but they have no effect on GABA release, this despite the presence of CB1 receptors on GABA terminals (Di et al., 2005a). Indeed, exogenously applied cannabinoids are equally effective at suppressing both glutamate and GABA release from excitatory and inhibitory synapses onto the magnocellular neurons, indicating that both glutamate and GABA synapses express functional CB1 receptors. This suggests, therefore, that the glucocorticoid-induced endocannabinoid actions are spatially restricted to glutamate synapses, and do not spill over onto neighbouring GABA synapses. Similarly, the unidentified retrograde messenger at GABA synapses causes a facilitation of synaptic GABA release, but has no effect on neighbouring glutamate synapses in the same neurons. Like the effect on glutamate release, this glucocorticoid action was abolished by blocking G protein activity specifically in the postsynaptic cell by applying a G protein antagonist via the patch pipette, which suggests that there was no spillover of the retrograde messenger released from adjacent cells that were also exposed to the bath-applied glucocorticoids. Therefore, although we do not yet know whether glutamate synapses also respond to the unknown retrograde messenger released at GABA synapses, we can conclude that the actions of this

messenger are nevertheless spatially restricted to the synapses on the cell in which the messenger is synthesized, i.e., there is no spillover of the messenger between cells. Thus, this indicates that there is a strict synapse-specific segregation of the actions of the retrogradely released messengers at glutamate and GABA synapses on the same postsynaptic cells, this despite the close spatial association of these synapses (Fig. 5). These steroid signalling mechanisms are novel not only in that they are mediated by rapid, non-genomic actions of the glucocorticoids, but also because they invoke a non-classical form of neurotransmission and multiple retrograde messengers selective for different types of synapses. Further investigation will address the underlying mechanisms of this synapse-specific spatial segregation of the glucocorticoid regulation of the magnocellular neurons.

By decreasing glutamate release and facilitating GABA release, glucocorticoids would be expected to cause a marked reduction in the excitability of these cells in vivo. Stress and stress levels of glucocorticoids have been shown to inhibit vasopressin and oxytocin release, to suppress the milk ejection reflex in lactating animals, and to delay parturition in late pregnancy (Douglas and Russell, 1994), and the rapid actions of glucocorticoids on the magnocellular neurons described here may play an important role in the stress inhibition of oxytocin function. Glucocorticoids have also been found to modulate vasopressin function under conditions of stress, for example during haemorrhage. Haemorrhage is a robust stressor that causes a large increase in circulating glucocorticoids, as well as an increase in both neurohypophysial vasopressin release into the blood stream and sympathetic norepinephrine release. The elevated circulating glucocorticoids have been found to exert a restraining influence on the haemorrhage-induced elevation of vasopressin and norepinephrine in the blood (Darlington et al., 1989). The inhibitory effect of glucocorticoids on blood vasopressin and norepinephrine levels is presumably via an acute, central effect of the elevated glucocorticoids, thus invoking the rapid glucocorticoid-induced, endocannabinoid-mediated suppression of synaptic activation of neurohypophysial vasopressin neurons (Di et al., 2005b) and possibly of descending preautonomic neurons controlling sympathetic norepinephrine release (Zsombok et al., 2007). When adrenalectomy was combined with fasting, the response to haemorrhage was found to be fatal, which was attributed not to the fasting-induced hypoglycemia, since it was not prevented by glucose replacement, but to hepatic failure caused by the vasoconstriction due to the unrestrained high levels of circulating vasopressin and norepinephrine (Darlington et al., 1990). Thus, the stress-induced circulating glucocorticoids would be responsible for restricting the secretion of vasopressin and norepinephrine via a rapid feedback mechanism, thereby preventing an exaggerated, potentially fatal vasoconstriction.

In summary, we have found a rapid glucocorticoid suppression of glutamate synaptic inputs and facilitation of GABA synaptic inputs to magnocellular neuroendocrine cells of the PVN and SON mediated by the actions of distinct retrograde messengers that target specifically each type of synapse. The effect of the rapid glucocorticoid synaptic regulation should mediate an overall inhibition of the magnocellular neurons, providing a cellular mechanism for the rapid inhibition of oxytocin and vasopressin function by stress. It will be interesting in the future to study the plasticity in the oxytocin neuron sensitivity to rapid actions of glucocorticoids in light of the desensitization of oxytocin function to stress activation during lactation.

Abbreviations

AEA	anadamide
2-AG	2-arachidonoylglycerol
CB	cannabinoid
CRH	corticotropin releasing hormone
GABA	γ-aminobutyric acid
HPA	hypothalamic–pituitary–adrenal
MAP kinase	mitogen-activated protein kinase
mEPSC	miniature excitatory postsynaptic current
mIPSC	miniature inhibitory postsynaptic current
NO	nitric oxide
PDE	phospodiesterase

PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PVN	paraventricular nucleus
SON	supraoptic nucleus

Acknowledgements

We thank Dr. Renato Malcher-Lopes for his dedication and contribution to this study, and Katalin Halmos for her expert technical assistance with the study.

This work was supported by National Institutes of Health Grants MH066958 and MH069879.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 32

Vasopressin in the septum: not important versus causally involved in learning and memory — two faces of the same coin?

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Abstract: Intraseptal arginine vasopressin (AVP) has been suggested to control in laboratory rodents not only emotionality but also learning and memory. However, depending upon the nature of the test procedure and, thus, the specific memory paradigm under study, administration of synthetic AVP into the lateral septum can have no effect, enhance or even impair learning and memory. Similar contradictory results were obtained after local administration of AVP V1 receptor antagonists in different learning and memory paradigms: blockade of AVP signalling in the lateral septum revealed either no essential function or a significant contribution of the endogenous neuropeptide. Based on the data available from studies investigating the impact of AVP in classical and operant conditioning, olfactory recognition and Morris water maze learning, it is proposed that endogenous AVP released within the lateral septum acts as neurotransmitter and neuromodulator to favour elemental (mono-modal) over complex (multi-modal) stimulus processing. Excessive availability of AVP, for example by intraseptal administration of the synthetic neuropeptide, interferes with memory performance in such tasks in which the integration of complex stimuli by the dorsal hippocampus is required, most likely by an inhibition of the septo-hippocampal pathway. In contrast, performance in tasks which focus on the processing of elemental stimuli such as olfactory recognition and classical and operant conditioning can be improved by intraseptal AVP administration, presumably due to an attenuation of complex stimulus processing.

Keywords: vasopressin; septum; hippocampus; learning; memory

Sources and targets of intraseptal AVP

In laboratory rodents immunohistochemical screening outside of the hypothalamus reveals a particularly high number of axons containing arginine vasopressin (AVP) within the lateral septal brain area. Different studies confirmed that these axons originate from the bed nucleus of the stria terminalis and the amygdala (De Vries and Buijs, 1983; Caffe et al., 1987). Interestingly, the intensity of the vasopressinergic innervation differs between the genders and is determined within a relatively short-lasting time window during ontogenesis by the amount of circulating steroids (De Vries et al., 1983) with male rodents showing a higher density of vasopressin-immunoreactive fibres than female animals (De Vries et al., 1983; Wang et al., 1998). In addition, both receptor-binding studies and in situ

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DOI: 10.1016/S0079-6123(08)00432-9

hybridization experiments revealed a high density of AVP-binding sites confined to the lateral septum which seem to consist almost exclusively of the V1a receptor subtype (Raggenbass et al., 1987). There is good evidence that in the septum, AVP acts as a neurotransmitter within the synaptic cleft (Raggenbass et al., 1988; Van den Hooff and Urban, 1990; Jacab and Leranth, 1995). Thus, it provides an intimate signal between two neurons as a very fast and selective messenger. However, as seen for all neuropeptides, the localization of the receptors does not entirely match with localization of AVP containing fibres and somata. This implies that neuromodulation is an important function of this neuropeptide in inter-neuronal communication (Landgraf and Neumann, 2004). Indeed it has been suggested that AVP and the closely related nonapeptide OXT may travel up to centimetres within the extracellular fluid in biologically relevant concentrations, bind to extra-synaptically located receptors and induce effects (Landgraf and Neumann, 2004).

Stimuli for intraseptal AVP release

The action of AVP as signal to transfer information between neurons requires not only the presence of the neuropeptide within neuronal structures and receptors in their vicinity but also AVP release into the extracellular space, for example after behaviourally relevant stimulation. Microdialysis in conjunction with highly sensitive radioimmunoassay enabled to monitor the release of AVP within the septum during behavioural performance. Using these tools, it was demonstrated that an acute exposure to a 10 min forced swimming session in 20°C warm water triggers an increase in the extracellular AVP concentration within the mediolateral septum. It is important to note that this release profile was not mirrored within the cerebrospinal fluid (CSF) (Ebner et al., 1999). This supports the hypothesis that the increased intraseptal AVP levels result from local (axonal) release originating from neurons belonging to the steroid-dependent vasopressinergic system and not from a simple diffusion of the neuropeptide released within the suprachiasmatic nucleus via CSF (Ebner et al., 1999). Moreover, the possibility that under these acute conditions a parenchymal diffusion may have contributed to the AVP concentrations measured within the septum is rather unlikely as the release profiles obtained from SON and PVN differ in that they reach their peak levels after the swimming session and, thus, at a time point when intraseptal AVP concentrations already returned to basal levels (Wotjak et al., 1998). Finally, AVP levels measured in microdialysates obtained from the dorsomedial hypothalamus failed to show any fluctuations in response to forced swimming (Engelmann et al., 1998a), thereby strengthening the suggestion of a local release of AVP within the septum after appropriate stimulation. Interestingly, experience of a social defeat did not trigger intraseptal AVP release (Ebner et al., 2000). Instead, adult male responded to a defeat by a dominant cage resident with an increase in the release of the closely related nonapeptide oxytocin (Ebner et al., 2000). Taken together, these data show that an acute exposure to distinct challenging and stressful stimuli triggers the release of AVP or OXT within the septum from local neuronal structures that can be monitored via the microdialysis technique. However, it is worth noting that AVP signalling at the level of individual synapses escapes from the detection by microdialysis. Therefore, it seems likely that the increased AVP and OXT concentrations measured in microdialysates reflect an increase in AVP acting as neuromodulator rather than neurotransmitter (Wotjak et al., 2008).

AVP signalling in the septum affects learning and memory: three examples

Spatial navigation

The behavioural relevance of AVP signalling within the septum has been investigated in a variety of studies using very different behavioural paradigms. Among them the Morris water maze (MWM) has attracted particular interest as it requires the integrity of the dorsal hippocampus (Moser et al., 1993) and, thus, has been regarded to allow the investigation of declarative memory

processes in non-primate species. The standard procedure of this task consists of exposing animals to a circular water-filled pool that contains a submerged target. The animals are released into the water and trained to find the target. The more precisely the animals remember the exact target localization, the shorter are the swimming durations (escape latencies) and the swimming path. A precise target navigation requires a correct integration of complex visual cues to compare the actual position with desired position in the maze. The water serves as aversive stimulus that motivates the animals to search for the target. Administration of a V1 antagonist into the septum via microdialysis (Engelmann et al., 1992a) or osmotic minipumps (Everts and Koolhaas, 1999) during acquisition failed to affect MWM navigation. In contrast, administration of synthetic AVP interfered with spatial navigation as it increased the escape latencies (Engelmann et al., 1992a). Interestingly, mutant rats lacking the ability to process the precursor peptide for AVP due to a point mutation (Brattleboro rats) show — except for the first session — a normal MWM performance (Engelmann and Bureš, unpublished observations).

Recognition memory

Social recognition relies on the ability of laboratory rodents to identify conspecifics individually based on their "olfactory signature". In this task the individual recognition abilities are tested in two consecutive sessions which are separated by a defined inter-exposure interval. During the first session (sampling) the experimental animal is allowed to acquire the olfactory signature of a given conspecific that is re-exposed during the second session (choice). The spontaneous investigatory behaviour of the experimental subject towards the conspecific during sampling and choice serves as an index for recognition. If recognized, the previously encountered conspecific will be less intensively investigated than the novel, previously not encountered animal. This task provides insight into non-conditioned memory processes in rodents although also contextual elements are known to affect social recognition memory (Burman and Mendl, 1999). Interestingly, under laboratory conditions, rats are able to form only a relatively short-term recognition memory: male animals are able to remember a previously encountered conspecific normally no more than 60 min after sampling (Dantzer et al., 1987). Different studies reported an inhibitory effect of intraseptally administered V1 antagonists on the recognition memory of adult male rats (Dantzer et al., 1988; Engelmann and Landgraf, 1994; Everts and Koolhaas, 1997). In contrast, administration of synthetic AVP improved social recognition memory in rats of this gender (Dantzer et al., 1988; Engelmann and Landgraf, 1994). The impression of a facilitatory action of AVP in the lateral septum for consolidation and/or retrieval of recognition memory in male animals was further strengthened by studies using the anti-sense approach. Male rats, in which the number of V1a receptors was selectively knocked down by local infusion of anti-sense oligonucleotides showed impaired recognition memory (Landgraf et al., 1995a). Further insight into the role AVP signalling within the septal brain area was obtained by investigating social recognition memory in both castrated male and female animals. Both groups showed longer recognition abilities than males, lasting for approximately 120 min. Additional experiments provided evidence that castrates and female rats are insensitive to V1 antagonist treatment (Bluthe and Dantzer, 1990; Bluthe et al., 1990). These data suggest a sexually dimorphic role for AVP signalling in the septum. Indeed, additional studies proved that in female rats endogenous OXT rather than AVP may play an important role in social recognition memory (Engelmann et al., 1998b). In this context it is of interest to note that in male Brattleboro rats the congenital absence of AVP leads to an impaired recognition memory which could be restored by local infusions of the synthetic neuropeptide (Engelmann and Landgraf, 1994). Most striking, however, was the observation that AVP treatment improved the recognition abilities in female and castrated male rats although the endogenous neuropeptide in these animals does not seem to be involved in this type of learning and memory (Bluthe and Dantzer, 1990; Bluthe et al., 1990).

Conditioning

Conditioning refers to experimental setups in which the animals learn to associate a conditioned with an unconditioned stimulus. The conditioned stimulus is a ''neutral'', previously not encountered mono-modal stimulus such as a tone, light, odour or taste signal. The unconditioned stimulus is typically an electrical footshock and evokes, therefore, a behavioural response which is aversive. The animal has to learn that both stimuli are paired and that the presentation of the conditioned stimulus predicts that of the unconditioned stimulus. Classical conditioning experiments rely on the measurement of the pure innate aversive response (e.g. freezing behaviour) without allowing the animal to avoid the unconditioned stimulus (stimulus–stimulus association; elemental conditioning). Thus, the animal cannot control the unconditioned stimulus. In contrast, during (negatively reinforced) operant conditioning the animals can avoid the unconditioned stimulus actively or passively and thus gains control over it. Typical examples for operant conditioning are shuttle box learning and jumping onto a pole for active versus step-down or step-through for passive avoidance tasks.

Administration of synthetic AVP into the mediolateral septum via microdialysis in a model for operant conditioning (pole-jumping avoidance) failed to affect the acquisition of the stimulus association. However, local administration of a V1 receptor antagonist interfered with pole-jumping avoidance suggesting that the endogenous neuropeptide signalling within the septum facilitated stimulus–response associations (Engelmann et al., 1992b).

Desmedt et al. (1999) investigated the consequences of the administration of AVP versus a V1a receptor antagonist into the lateral septum of mice during classical conditioning. Mice were exposed to an unfamiliar environment and given a footshock either overlapping with a tone signal (conditioned stimulus) or separated from the tone by 20–30 s. It is expected that tone-shock overlapping enables the direct association between the two stimuli (so-called cued conditioning), whereas the temporal separation of the tone from the footshock facilitates the association ''new environment–footshock'' (so-called context conditioning). Whether or not the animals made one or the other or both associations can be tested during memory sessions: Exposure to the originally unfamiliar complex environment ''footshock chamber'' without tone presentation enables the measurement of context conditioning. Exposure to the tone in a new, previously not encountered environment provides access to the ''tone–footshock'' association. Freezing behaviour shown by the animals during the memory session serves as an index for the strength of the respective associations. The authors observed that intraseptal AVP treatment facilitated the tone–shock association if the two stimuli were temporally separated. At the same time it suppressed the otherwise typically observed context association. Interestingly, administration of an AVP antagonist did not affect the behaviour of the animals under these conditions, but impaired cued fear conditioning if tone and shock overlapped (Desmedt et al., 1999). These data imply that AVP released within the septum facilitates elemental (mono-modal) stimulus association and — at least in pharmacological dosages — interferes with complex stimulus processing.

Conclusion

As outlined above, AVP is released within the lateral septum under defined stimulus conditions to act not only as neurotransmitter but also as neuromodulator for controlling distinct types of learning and memory. In male animals axons of the steroid-dependent vasopressinergic system located in this brain area seem to release AVP to facilitate the processing of elemental, mono-modal stimuli such as acoustic or visual stimuli in case of classical conditioning or olfactory stimuli in case of social recognition. Interestingly, particularly excessive levels of AVP in the lateral septum induced for instance by administration of the synthetic neuropeptide seem to affect also the processing of complex stimuli by an inhibitory impact on the activity of the septo-hippocampal complex (Desmedt et al., 1999). In fact, in a recent paper the group of Raggenbass (Allaman-Exertier et al., 2007) showed

that AVP potentiates the inhibitory output of almost all GABAergic interneurones in the lateral septum via an interaction with dendritically and somatically located V1a receptors. This suggests that the stimulation of GABAergic interneurons by AVP in the lateral septum may result in an inhibition of the cholinergic output of the medial septum to the hippocampus.

How could a control of hippocampal activity by AVP signalling within the lateral septum affect the outcome in the different learning and memory tasks? A testable hypothesis may provide the evidence accumulated in studies investigating the neuronal substrate underlying classical conditioning (Fig. 1). Different experimental approaches revealed that the hippocampus binds separate stimuli of complex environments represented in the cortex into a new unit called conjunctive representation which is then used for further processing (Rudy et al., 2004). Such conjunctive representations are a conditio sine qua non to successfully solve tasks related to complex environments such as the MWM and contextual conditioning (Rudy et al., 2004). In contrast, tasks in which conjunctive representations and, thus, the hippocampus play an inferior role compared to the processing of

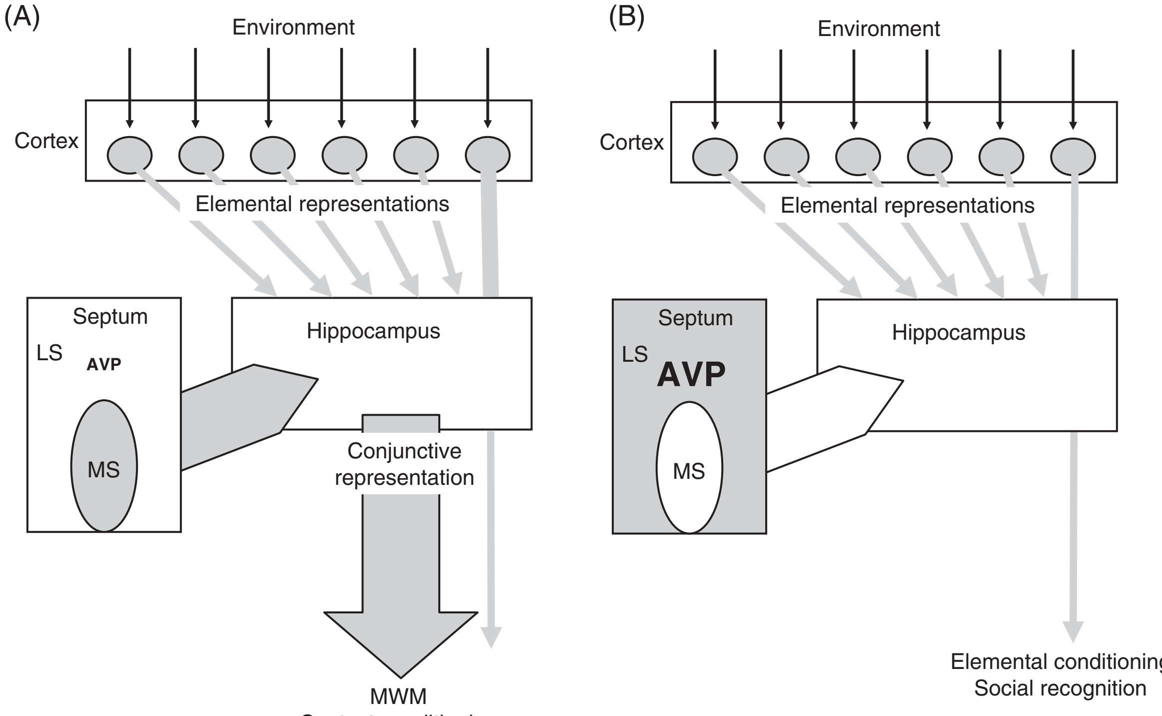

Fig. 1. Schematic drawings modified after Desmedt et al. (1999) and Rudy et al. (2004) illustrating the suggested role AVP plays in the lateral septum (LS) for controlling the interaction of cortex, hippocampus and medial septum (MS) to promote different types of learning and memory. Individual features of the environment (thin black arrows) are represented in the cortex (grey circles; elemental representations), can be retrieved by the hippocampus (grey arrows) and bound to a unit (conjunctive representation), which is further used for memory processing (big grey arrow). In parallel, elemental representations will be processed independently by other brain areas (e.g. amygdala). (A) Low AVP signalling in the LS leads to a high output of MS (grey) neurons to the hippocampus (grey arrow), where the assembly of conjunctive representations is promoted. Conjunctive representation may be essential to successfully solve the MWM and context conditioning. (B) High AVP levels in the LS (grey; e.g. by administration of the synthetic peptide) reduce the output of the MS to the hippocampus resulting in a low activity to assemble conjunctive representations. This results in a promotion of elemental over conjunctive representations for memory processing which may support elemental conditioning and social recognition memory (but interferes with MWM and contextual learning).

elemental stimuli should remain almost unaffected from hippocampus inactivation. Indeed, lesioning the hippocampus failed to interfere with social recognition memory in rats (Bannerman et al., 2001; Squires et al., 2006), and simple stimulus–stimulus associations can be made without the hippocampus being involved (Phillips and LeDoux, 1992).

The hypothesis that excessive AVP signalling in the lateral septum blocks the ability of the hippocampus to create conjunctive representations would explain why administration of the synthetic neuropeptide into the lateral septum interferes with the ability of rats to find the hidden target during MWM learning whereas blocking the endogenously released neuropeptide is without effect. Moreover, the otherwise difficult-to-explain observation that AVP administration is still able to improve social memory in both female rats and castrated males despite the absence of the endogenous neuropeptide in these animals may become plausible. However, the effect of intraseptally released AVP for learning and memory does not seem to be restricted to its action on hippocampal activity. Rather, the reported control of anxiety-related behaviour of the endogenous neuropeptide within the septum (Landgraf et al., 1995b; Liebsch et al., 1999; Bielsky et al., 2004) may provide a supportive mechanism to facilitate elemental stimulus processing by additional emotional colouring of incoming information.

The presented hypothesis may provide an explanation for the divergent observations concerning the role intraseptally released AVP of male animals plays for learning and memory. However, clearly, further studies are needed to test this hypothesis by investigating in more detail the impact of locally released AVP for the interplay between elemental and conjunctive stimulus representation and processing. These studies may also include an analysis of the interaction between the lateral septum and other brain regions including amygdala and cortical areas, which are well known to control the behavioural performance related to learning and memory.

Abbreviations

AVP	arginine vasopressin
MWM	Morris water maze

Acknowledgements

The author thanks Dr. Carsten Wotjak for critically reading the manuscript. During preparation of the manuscript the author received support by the following grant organisations: DFG (grants no. 436 UNG 113/168/0-2 and EN 366/6-1) and DAAD (grant no. D/07/09923).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 33

Role of the endocannabinoid system in regulation of the hypothalamic-pituitary-adrenocortical axis

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Abstract: The endocannabinoid system has been recognized as a major neuromodulatory system, which functions to maintain brain homoeostasis. Endocannabinoids are synthesized and released from the postsynapse and act as retrograde neuronal messengers, which bind to cannabinoid type 1 receptors at the presynapse. Here, they inhibit the release of neurotransmitters, including glutamate and GABA. By these means, endocannabinoids control the activation of various neuronal circuits including those involved in neuroendocrine stress processing. Accordingly, exogenous cannabinoids such as the major active component of marijuana, Δ^9-tetrahydrocannabinol, have long been known to activate the major neuroendocrine stress response system of mammals, the hypothalamic-pituitary-adrenocortical (HPA) axis. However, the function of the endocannabinoid system in the regulation of stress hormone secretion has only recently begun to be understood. It is the focus of the present review to provide the reader with an overview of our current knowledge of the role of endocannabinoid signalling in HPA axis regulation under basal as well as under stressful conditions. This includes the specific sites of action, potential underlying neuronal pathways and interactions between behavioural and neuroendocrine stress coping. Furthermore, the potential role of HPA axis activity dysregulations, caused by deficits in the endocannabinoid system, for the pathophysiology of psychiatric diseases is discussed.

Keywords: HPA; stress; endocannabinoids; CB1; corticosterone; ACTH; tetrahydrocannabinol; cannabinoids; rimonabant; depression; cortisol; GR; MR; feedback; pituitary; adrenal; THC

Introduction

Ever since the discovery of the cannabinoid type 1 (CB1) receptor (Matsuda et al., 1990), the first protein binding partner of the major psychoactive component of marijuana, Δ^9-tetrahydrocannabinol (THC), the investigation of CB1 receptor-mediated signalling within the brain has stimulated a tremendous amount of research. Because CB1 receptors are among the most widely distributed and abundantly expressed G-protein-coupled receptors in the brain (Herkenham et al., 1990), it is not surprising that CB1 receptor signalling has profound effects on a wide variety of behavioural, vegetative and autonomic functions (Di Marzo et al., 2005). The discovery of endogenous ligands for CB1 receptors (so-called endocannabinoids; Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995) paved the way for the understanding of the endocannabinoid system as a major

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DOI: 10.1016/S0079-6123(08)00433-0

neuromodulatory system of the brain, which influences the activity of neuronal circuits by inhibiting the release of a number of excitatory and inhibitory neurotransmitters (Piomelli, 2003; Degroot and Nomikos, 2007). Thus, endocannabinoid signaling can be expected to influence neuronal stress processing. However, even though the effects of exogenous cannabinoids on stress hormone secretion are known for many years, the role of endocannabinoid signalling for neuroendocrine stress processing has only recently begun to be deciphered (Carrier et al., 2005; Pagotto et al., 2006).

In the present review we highlight current evidence for the presence of different players of the endocannabinoid system at sites involved in hypothalamic-pituitary-adrenocortical (HPA) axis regulation. We then introduce pharmacological, genetic and neurochemical/neuroendocrine means by which exogenous and endogenous cannabinoid actions on HPA axis activity have been investigated in the past and offer perspectives for future research, followed by a compilation of the data on endocannabinoid-mediated stress hormone secretion. In this context we will provide a detailed overview of our current understanding of the role of endocannabinoid signalling for HPA axis activity under basal and stressful conditions. Finally, we summarize evidence for potential endocannabinoid-mediated interactions between behavioural and neuroendocrine stress coping, and we invite the reader to speculate on the potential role of endocannabinoid-mediated HPA axis disturbances for the development of neuropsychiatric disorders such as major depression.

The endocannabinoid system

Endocannabinoids are lipophilic arachidonic acid derivatives, which are primarily synthesized at the postsynapse (Di Marzo et al., 2005; Basavarajappa, 2007), from where they are released to reach across the synaptic cleft to bind to presynaptically located CB1 receptors (Piomelli, 2003). Receptor binding causes a wide variety of intracellular signalling mechanisms (Diaz-Laviada and Ruiz-Llorente, 2005), which, in the first place, serve to reduce the excitability of the presynapse and, in consequence, to reduce neurotransmitter release. In particular, CB1 receptor binding leads to inhibitory $G_{i/o}$-protein-mediated opening of inwardly rectifying potassium (K_{ir}) channels and closing of N- or P/Q-type Ca^{2+} channels. Also coupling to mitogen-activated protein kinase (MAPK) and akt thymona viral oncogene (Akt) signalling, as well as to the phosphoinositol pathway has been reported (Demuth and Molleman, 2006). Following release and CB1 receptor binding, endocannabinoids are prone to putative endocannabinoid transporter-mediated re-uptake into synaptic terminals and possibly surrounding glia cells, where they are rapidly degraded (De Petrocellis et al., 2004; Pazos et al., 2005). Several different endocannabinoids have been isolated until today. However, two of them have been most intensively studied, and they are referred to as the two major endocannabinoids, *N*-arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG) (Piomelli, 2003). Although examples for a tonic activation of CB1 receptors under basal conditions exist (Caille et al., 2007; Cota et al., 2007; Bequet et al., 2007; Oliet et al., 2007), endocannabinoids are thought to be primarily synthesized on demand in an activity-dependent manner (Piomelli, 2003). Following synthesis, endocannabinoids are transported through facilitated diffusion across the cell membrane by a putative endocannabinoid transporter, whose existence is suggested by increasing biochemical evidence, although the protein itself has not yet been cloned (Glaser et al., 2005; Hermann et al., 2006). Anandamide and 2-AG are synthesized and degraded by different enzymatic machineries, which tightly control their cellular concentration. For a detailed overview of these enzymatic steps we refer the reader to excellent reviews on this topic (Piomelli, 2003; Bisogno et al., 2005; Basavarajappa, 2007). Out of brevity reasons, we will just introduce the two major endocannabinoid degrading enzymes, fatty acid amide hydrolase (FAAH), which degrades anandamide (Schmid et al., 1995; Hillard et al., 1995; Ueda et al., 1995; Cravatt et al., 1996), and monoacyl glycerol lipase (MGL), which degrades 2-AG (Dinh et al., 2002). It is of importance to note that FAAH is primarily found in postsynaptic terminals (Tsou et al., 1998b;

Egertova et al., 2003), whereas MGL seems to be predominantly located in presynaptic terminals (Dinh et al., 2002). This supports the idea that the lifetime of both endocannabinoids may be differentially regulated in order to serve different functions.

The hypothalamic-pituitary-adrenocortical (HPA) axis

Stress is a highly adaptive response of an organism to challenges (i.e. stressors) in the environment that is aimed at re-establishing homoeostasis (for detailed discussions of stress concepts, see Chrousos and Gold, 1992; Pacak and Palkovits, 2001; Engelmann et al., 2004; McEwen, 2007). In general, researchers distinguish between two different types of stressors, (i) ‘‘physical stressors’’, where the organism is directly exposed to a physical (systemic) threat, such as changes in cardiovascular tone, somatic pain or inflammation, and (ii) ‘‘psychological stressors’’, where no actual physical challenge arises, but only the threat of danger exists, for instance, innate fear of an animal to predator odour or open and unprotected places (Herman et al., 2003). However, in nature even psychological stressors (e.g. predator odour) are very likely to result in physical demands (e.g. release of energy reservoirs to provide the basis for flight or fight), and this might be the reason behind the fact that both psychological and physical stressors induce similar major stress responses. Thus, the response of vertebrates to all kinds of stressor involves, although to different extents, (i) the activation of the HPA axis, which culminates in the release of glucocorticoids from the adrenal cortex into the blood circulation, and (ii) the activation of the sympatho-medullo-adrenal (SMA) axis (also known as sympatho-adrenergic system, SAS), which results in the release of noradrenaline and adrenaline from nerve terminals and the adrenal medulla, respectively, into the blood stream. Although the ultimate stress response (release of glucocorticoids and catecholamines from the adrenals) to both psychological and physical stressors is very similar, brain regions responsible for the initial integration of the different stressors seem to vary considerably. Whereas accumulating evidence speaks for a strong involvement of higher limbic brain regions such as hippocampus, amygdala and prefrontal cortex, in the interpretation of psychological stressors (Herman et al., 2005), mainly mid-hindbrain regions including monoaminergic brain nuclei, such as the locus coeruleus and the raphe nuclei, have been suggested to be responsible for the response to physical stressors (Herman et al., 2003).

After the initial processing and integration of threatening stimuli, afferent fibres from both limbic and mid-hindbrain regions converge onto parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus. They trigger the release of corticotropin-releasing hormone (CRH) form axon terminals of these neurons at level of the median eminence into the capillaries of the pituitary portal vessels that target the anterior lobe of the pituitary. Here, CRH binds to CRH type 1 receptors (CRH-R1) present in corticotrope cells of the anterior pituitary, driving the synthesis of adrenocorticotropic hormone (ACTH) from the precursor protein pro-opiomelanocortin (POMC) and its secretion into the blood stream. ACTH, in turn, is transported via the blood to the adrenocortical cells of the zona fasciculata of the adrenal glands, where it binds to ACTH receptors (melanocortin type 2 receptors, MC2-R) and stimulates the production and release of glucocorticoids (cortisol in humans, corticosterone in rodents) into the blood circulation.

It is obvious that an adequate corticosterone response to a stressor in terms of amplitude and duration is an extremely complex process, which needs to be regulated at many different sites. Therefore, although the primary brain structure responsible for the effect size of corticosterone secretion seems to be the PVN (Herman et al., 2003), further fine tuning of the corticosterone response at the level of the pituitary, where the CRH signal is integrated into ACTH release (Rivier et al., 1982; Engelmann et al., 2004), and at the level of the adrenals, where the ACTH signal is integrated into corticosterone release (Ehrhart-Bornstein et al., 1998), occurs. The picture is further complicated by the fact that, besides CRH, other neuropeptides, such as arginine–vasopressin

(AVP) and oxytocin (OXT) may act as secretagogues of ACTH as well. These peptides were shown to act synergistically with CRH to stimulate ACTH release from the pituitary (for review see Antoni, 1993) even if they are of magnocellular origin, i.e., originate from that neuronal population of the PVN and the supraoptic nucleus, which comprises the central part of the hypothalamic-neurohypophysial system (Engelmann et al., 2004). Also a potential interaction between SMA and HPA axes deserves further attention, since the SMA axis is known to affect HPA axis activity by modulating the responsiveness of the adrenal cortex to ACTH (Ehrhart-Bornstein et al., 1998; Ishida et al., 2005; Ulrich-Lai et al., 2006).

Once a corticosterone response has been mounted, there is the need for efficient negative feedback in order to ensure the adequate re-setting of the HPA axis following stressor termination. This corticosterone-mediated feedback is thought to occur at various sites, mainly the PVN and the pituitary (De Kloet et al., 2005; Armario, 2006). Some evidence also speaks for an involvement of the hippocampus (Jacobson and Sapolsky, 1991), and possibly the adrenal glands (unpublished observations from our laboratory). Glucocorticoids are known to act primarily through glucocorticoid receptors, which, in their role as classical transcription factors, dimerize upon binding, translocate into the nucleus and initiate the transcription of various stress response genes (De Kloet et al., 2005). Two classical glucocorticoid receptors are known, the type 1 glucocorticoid receptor, referred to as mineralocorticoid receptor (MR), and the type 2 glucocorticoid receptor (GR). MR is known to have about a 10-fold higher affinity for corticosterone than GR. Hence, MRs are mostly occupied during resting conditions, whereas GRs become only substantially activated during the dark, active period in rats and mice, when basal glucocorticoid levels rise, or after exposure to stress (De Kloet, 2003). Therefore, it is believed that mainly GRs are involved in the stress-induced corticosterone-mediated negative feedback. However, one has to keep in mind that GRs function primarily through their effects on transcription. Such processes might be responsible for delayed feedback, but cannot account for the fast-feedback HPA axis regulation, which occurs within minutes after stressor exposure. Although the evidence for these fast-feedback mechanisms of corticosterone is overwhelming (Dallman, 2005), the underlying molecular mechanisms are still poorly understood. Fast effects of corticosterone on ACTH release may be ascribed either to corticosterone-mediated actions on (i) a putative novel membrane-bound G-protein-coupled glucocorticoid receptor, (ii) cell membrane-bound fractions of GR or MR, (iii) to direct actions of glucocorticoids at the cell membrane or (iv) to cytosolic GR-mediated signal transduction pathways that are initiated before the transcription factor enters the nucleus (Dallman, 2005; Johnson et al., 2005; Karst et al., 2005; Maier et al., 2005; Song and Buttgereit, 2006). Recent data suggest that endocannabinoids may play a pivotal role in feedback regulation of HPA axis activity at level of the PVN primarily via membrane-bound G-protein-coupled glucocorticoid receptors (Di et al., 2003; Malcher-Lopes et al., 2006).

Means of studying the endocannabinoid system

Table 1 summarizes current research strategies aimed at investigating the physiological role of the endocannabinoid system of the brain. Initially, studies monitored consequences of exogenous CB1 agonists, such as THC, whose structure was delineated

Table 1. Means of investigating the function of the endocannabinoid system of the brain (selection)

CB1 receptor agonists	
	Δ^9-THC, HU-210, CP-55,940, WIN-55,212-2
CB1 receptor antagonists/reverse agonists	
	SR141716 (also known as Rimonabant), AM251, AM281
Endocannabinoid re-uptake inhibitors	
	AM404, UCM707, VDM11, OMDM1, OMDM2
Inhibitors of the anandamide degrading enzyme FAAH	
	URB597, URB592
Genetic models	
	Conventional CB1 receptor knockout and FAAH knockout mice; conditional CB1 receptor knockout mice
Measurement of endocannabinoid levels	
	Brain punches, microdialysis approaches

in 1964 (Gaoni and Mechoulam, 1964), or later of synthetic CB1 agonists, including WIN-55,212-2, CP-55,940 and HU-210, which were generated in the early 1990s (Howlett et al., 2002). Application of exogenous CB1 agonists induces a ubiquitous activation of CB1 receptors throughout the brain in an indiscriminative manner.

Recently a number of pharmacological and genetic tools have been established, which allow to abolish endocannabinoid signalling via CB1 receptors. To present, four different lines of CB1 receptor knockout mice were generated [Ledent et al., 1999 (CD1 background); Zimmer et al., 1999 (C57BL/6J background); Marsicano et al., 2002 (C57BL/6N background); Robbe et al., 2002 (C57BL/6 background)], three of which have already been studied with regard to HPA axis activation. One of those mutant lines deserves particular attention as it has been developed as a conditional knockout using the Cre/loxP system (Marsicano et al., 2002). Thus, crossing this line with different Cre recombinase expressing transgenic lines, as done, for instance, in context of kainic acid-induced seizures (Marsicano et al., 2003; Monory et al., 2006), may allow deeper insights into the brain region- and cell type-specific involvement of CB1 receptors in HPA axis regulation. Apart from these genetic tools, a class of CB1 receptor antagonists/inverse agonists has been synthesized including SR141716 (also known as Rimonabant; Rinaldi-Carmona et al., 1994), AM251 (Gatley et al., 1996) and AM281 (Lan et al., 1999).

Other approaches attenuate uptake and degradation of endocannabinoids, thus promoting endocannabinoid signalling specifically in those brain areas, where the endocannabinoid system is intrinsically active. Firstly, a FAAH-knockout line was generated (Cravatt et al., 2001), which shows highly elevated levels of anandamide, leaving 2-AG level fairly unaffected. To our knowledge, this mouse line has not been investigated yet in respect to HPA axis activity. However, it may prove useful in the future in delineating the differential contribution of the two major endocannabinoids, anandamide and 2-AG, for HPA axis regulation. Secondly, a class of FAAH inhibitors has been developed, including URB597 and URB592 (Kathuria et al., 2003), which specifically enhance anandamide levels. Thirdly, there is a class of endocannabinoid transport inhibitors, which includes AM404 (Beltramo et al., 1997), UCM707 (Lopez-Rodriguez et al., 2003), VDM11 (De Petrocellis et al., 2000), OMDM-1 and OMDM-2 (Ortar et al., 2003). These inhibitors enhance anandamide and 2-AG signalling specifically at the sites of intrinsic activity by blocking their uptake into nerve terminals and glia cells, thus increasing their availability at CB1 receptors.

Importantly, biological functions of the endocannabinoid system can be assessed not only by modifying endocannabinoid signalling by pharmacological or genetic means, but also by directly measuring the activity status of the endocannabinoid system, for instance by quantification of endocannabinoid levels or CB1, MGL and FAAH activity (Bortolato et al., 2007; Rademacher et al., 2008). Endocannabinoid levels are typically determined in tissue samples. Due to the lipophilic nature of endocannabinoids, only a few studies have so far achieved to successfully employ the microdialysis technique for monitoring dynamic changes in endocannabinoid release in response to pharmacological challenges under in vivo conditions (Giuffrida et al., 1999; Walker et al., 1999; Bequet et al., 2007; Caille et al., 2007). Although quantification of endocannabinoids in brain punches represents a major technical achievement, there are several caveats if it comes to the interpretation of the data: Firstly, tissue samples do not allow differentiating between extracellular (and supposedly, biologically active) and intracellular endocannabinoid pools. In fact, whereas levels of 2-AG from brain punches are three magnitudes larger than those of anandamide, extracellular endocannabinoid levels assessed by microdialysis procedures differ by less than one magnitude (Bequet et al., 2007; Caille et al., 2007). Secondly, endocannabinoid levels show rapid and divergent dynamic changes (De Lago et al., 2005; Hohmann et al., 2005; Rademacher et al., 2008), partially because endocannabinoids are synthesized and released on demand and are not stored in synaptic vesicles, and these temporal changes are easily missed when analysing tissue levels. Thirdly, 2-AG "emerges" also in the context of a variety of metabolic and catabolic pathways unrelated to the endocannabinoid system.

Characteristics of the endocannabinoid system at different levels of the HPA axis

CB1 receptors are expressed in high abundance in the brain. They are prominently present in many limbic brain regions involved in HPA axis regulation, such as the prefrontal cortex, hippocampus and amygdala (Herkenham et al., 1990, 1991; Matsuda et al., 1993; Tsou et al., 1998a; Pettit et al., 1998). The receptor is also expressed in subcortical regions, such as the bed nucleus of the stria terminalis (BNST) and the PVN, however, at much lower levels (Herkenham et al., 1991; Romero et al., 1998; Tsou et al., 1998a; Wittmann et al., 2007). In mid-hindbrain regions, CB1 receptor expression is sparse (Herkenham et al., 1991; Tsou et al., 1998a). Recent evidence suggests faint CB1 receptor expression in monoaminergic nuclei, which may be involved in regulating the HPA axis response to physical stressors. Accordingly, CB1 receptors were found in serotonergic (Haring et al., 2007) and noradrenergic (Oropeza et al., 2007) neurons.

Originally thought to be mainly localized in GABAergic neurons, latest findings strongly suggest localization of the CB1 receptor in glutamatergic neurons as well (Marsicano and Lutz, 1999; Marsicano et al., 2003). In this regard, it is remarkable that, even though CB1 receptor expression in glutamatergic neurons is generally much lower than in GABAergic neurons, it is often precisely CB1 receptor signalling in these glutamatergic neurons, which is responsible for the manifestation of certain phenotypes, such as protection against kainic acid-induced seizures (Marsicano et al., 2003; Monory et al., 2006) or some typical behavioural responses to THC (Monory et al., 2007). In addition, CB1 receptor-mediated reduction of glutamate release from glutamatergic synapses, which innervate and activate parvocellular neurons of the PVN, is held responsible for glucocorticoid-mediated fast-feedback regulation of the HPA axis (Di et al., 2003).

In light of the importance of the CRH system for the hormonal stress response it is reasonable to investigate its potential interaction with the endocannabinoid system. Recent double in situ hybridization studies showed that CB1 receptor mRNA is co-localized with CRH mRNA not only in more than 50% of CRH positive neurons in the hypothalamic nuclei of the PVN (Cota et al., 2003), but also in several extrahypothalamic areas, such as the amygdala, prefrontal cortex and BNST, which are known to be involved in stress integration (Cota et al., 2007). On basis of these studies it is tempting to speculate that CB1 receptor signalling at the presynaptic site might be involved in regulating the neuronal release of CRH in limbic brain areas as well as possibly at the level of the median eminence. It remains to be mentioned that CB1 receptors have also been shown to co-localize with CRH type 1 receptors (CRH-R1) in neurons, which express low levels of CB1 and which are likely of glutamatergic nature (Hermann and Lutz, 2005). This co-expression, however, does not necessarily imply a direct interaction between the two receptor systems, in particular if one considers that CB1 receptors are expressed primarily presynaptically, whereas CRH-R1s are thought to be localized postsynaptically (Chen et al., 2004).

CB1 receptors can also be found in the pituitary gland. After initial radioligand binding studies, which revealed only very sparse receptor expression in the rat pituitary (Herkenham et al., 1991; Lynn and Herkenham, 1994), subsequent in situ hybridization and immunohistochemistry studies could clearly identify CB1 receptor mRNA and protein in the rat anterior pituitary and the intermediate lobe, but not in the neural lobe (Gonzalez et al., 1999; Wenger et al., 1999). Later, CB1 receptor expression was also found in the pituitary of *Xenopus laevis* (Cesa et al., 2002), *Rana esculenta* (Meccariello et al., 2006), *Pelvicachromis pulcher* (Cottone et al., 2005) and *Homo sapiens* (Galiegue et al., 1995; Pagotto et al., 2001), suggesting that its presence in the pituitary gland is conserved among different species. However, the pituitary cell types, in which CB1 receptors are expressed, differ considerably between different species. Whereas CB1 receptors in humans are localized in somatotrophs, lactotrophs, corticotropes and folliculostellate cells (Pagotto et al., 2001), CB1 receptors in the rat and frog pituitary are localized mainly in gonadotrophs and lactotrophs and in frogs to some extent in thyrotrophs, but CB1 receptors are largely absent from corticotropes in both rats and

frogs (Wenger et al., 1999; Cesa et al., 2002). Pagotto et al. (2001) demonstrated in human coricotropinoma primary cells that a co-stimulation of CB1 receptors by a CB1 agonist had an additive effect on CRH-stimulated ACTH secretion, suggesting that CB1 receptor signalling in the human pituitary may actually aid in regulating ACTH release. In rodents, however, this scenario seems unlikely, because CB1 receptors are not expressed in corticotropes (Wenger et al., 1999). In support of this notion, Barna et al. (2004) did not find any effect of the genetic loss of CB1 receptors on CRH-mediated ACTH secretion from pituitary ex vivo explants of CB1 receptor knockout mice. On the other hand, we could recently show that pituitary primary cultures of CB1 receptor knockout mice reacted with an increased ACTH release upon stimulation with CRH in vitro (Cota et al., 2007). In light of these discrepant findings it seems inevitable to establish an in vivo experimental setup to clarify the role of CB1 receptor signalling for ACTH release in the rodent pituitary of the intact organism.

In addition to CB1 receptor expression, also the synthesis of endocannabinoids has been established for the pituitary gland. Considerable levels of the two major endocannabinoids, 2-AG and anandamide, were found in the rat and human pituitary (Gonzalez et al., 1999; Pagotto et al., 2001) and the influence of sex steroids on endocannabinoid synthesis could be demonstrated in the rat (Gonzalez et al., 2000; Bradshaw et al., 2006).

It remains to be mentioned that, although CB1 receptor expression was found being virtually absent in the nervous part of the median eminence in an early radioligand binding study (Herkenham et al., 1991), CB1 receptor immunoreactivity could recently be established in axon terminals of the murine median eminence by means of a novel CB1 receptor antibody directed against its C-terminus (Wittmann et al., 2007). Therefore, it seems possible that CB1 receptor protein, which is translated from CB1 receptor mRNA in parvocellular CRH-containing neurons of the hypothalamus (Cota et al., 2003), may regulate CRH secretion from these neurons into the hypophyseal portal blood at level of the median eminence. If CB1 receptors are indeed present at nerve terminals in the median eminence, it is reasonable to ask for the origin of endocannabinoids which may bind to these receptors. In this context, Wittmann et al. (2007) suggested that they might be released either from the very same nerve terminals to act via an autocrine mechanism, or from the surrounding vascular endothelial cells and/or astrocytes and tanycytes to act in a paracrine manner. However, one study from the late 1990s argues against a physiological significance of CB1 receptors expressed on nerve terminals of CRH neurons at the level of the median eminence, demonstrating that anandamide is able to selectively stimulate the release of gonadotropin-releasing hormone release, but not of CRH, from rat median eminence explants (Prevot et al., 1998).

In contrast to the pituitary, much less is known about the endocannabinoid system in the adrenal glands. Just one study exists in humans, where only a very faint CB1 mRNA signal was detected in the adrenals by real-time PCR (Galiegue et al., 1995). In rats, CB1 receptor expression has just been studied during development. It could be shown that CB1 receptor mRNA expression, as detected by in situ hybridization, seems to shift from the adrenal cortex at stage E17 to the zona glomerulosa at E20 (Buckley et al., 1998). In the adrenal glands of adult rabbits, in contrast, neither an in situ hybridization signal, nor radioligand binding could be detected (Niederhoffer et al., 2001). Because the authors found a cannabinoid-mediated inhibition of adrenaline secretion from isolated rat adrenal glands despite the aforementioned lack of CB1 expression, they suggested that this effect is likely mediated via CB1 receptor-mediated inhibition of acetylcholine release from preganglionic sympathetic nerve terminals (Niederhoffer et al., 2001). So far, it is not known whether or not endocannabinoid signalling also influences ACTH-mediated corticosterone secretion directly at level of the adrenal cortex.

Effects of exogenous cannabinoids on HPA axis activity

The stimulatory effects of exogenous cannabinoids on HPA axis activity are known for many decades

and have been thoroughly investigated. Dewey et al. (1970) have been among the first who could demonstrate that intraperitoneal (i.p.) injection of THC decreased ascorbic acid content in the rat adrenal gland as an index of increased ACTH secretion. One year later, Kubena et al. (1971) showed that the i.p. injection of THC increases plasma corticosterone in the rat in a dose-dependent manner, which was judged a more precise measure of HPA axis activation. The authors, furthermore, showed that hypophysectomy completely blocked the adrenocortical response to THC, suggesting that the drug rather acts on brain structures stimulating the pituitary than on the adrenal cortex per se (Kubena et al., 1971). However, it was not before 1982, around the time when radioimmunoassays for ACTH had become available, that Puder and co-workers could demonstrate the direct activation of ACTH secretion by THC. Furthermore, by applying complete hypothalamic deafferentation, according to the method of Halasz and Gorski (1967), these authors demonstrated that THC-mediated effects on ACTH and corticosterone secretion must be mediated through effects on extrahypothalamic brain regions rather than via direct stimulation of ACTH release at level of the hypothalamus, median eminence and/or pituitary gland or of corticosterone synthesis at the level of the adrenal gland (Puder et al., 1982). These findings are supported by later studies demonstrating that the intracerebroventricular administration of THC is sufficient to induce ACTH and corticosterone secretion (Weidenfeld et al., 1994; Manzanares et al., 1999).

As summarized in Table 2, most of the studies showed a robust stimulatory effect of THC on corticosterone secretion, which was independent (i) of the species (as it could be observed in mice, rats, cattle and humans), (ii) of sex and (iii) of the route of administration (intracerebroventricular, i.p., subcutaneous, intravenous and, with a couple of exceptions, also oral administration). Furthermore, the effects of THC were (iv) dose-dependent (with effective doses being greater than 1 mg/kg and an inverse U-shaped dose–response relationship at very high concentrations; Kubena et al., 1971; Johnson et al., 1978; Zuardi et al., 1984), (v) rapid in onset (with elevated corticosterone levels 30 min after i.p. injection; Schramm-Sapyta et al., 2007) and (vi) long lasting (up to 5 h; Newton et al., 2004). Furthermore, (vii) the stimulation of HPA axis activity seems to be universal for all kinds of CB1 receptor agonists, as also the exogenous cannabinoids 11-OH-Δ^9-THC (Johnson et al., 1978), CP-55,940 (Romero et al., 2002) and HU-210 (Martin-Calderon et al., 1998), as well as the endocannabinoid anandamide (Weidenfeld et al., 1994; Wenger et al., 1997; Zenor et al., 1999) were shown to increase corticosterone secretion. Overall, a similar picture as for corticosterone also applies to ACTH, even though ACTH plasma levels were determined in a fewer number of studies than corticosterone (compare Table 2).

It is noteworthy that CB1 receptor agonists increased ACTH and corticosterone secretion not only under basal conditions (or better, after mild injection stress, because the injection of a drug inevitably confers some stress to the animal; except for drug administration via chronically implanted jugular venous catheters), but also in consequence of a confrontation with more intense stressors. Accordingly, CB1 receptor agonists were shown to further increase stress-induced corticosterone secretion in response to exposure to the tail immersion test followed by the holeboard (Romero et al., 2002), to the holeboard followed by the elevated plus maze (Marco et al., 2006), to the open field with concomitant presentation of ultrasound noise (Finn et al., 2004a) and to inescapable electric foot shocks (Jacobs et al., 1979). However, in response to ether stress or lipopolysaccharide (LPS) injection, no further elevation of corticosterone secretion was observed (Dalterio et al., 1981; Roche et al., 2006), possibly because of ceiling effects. One recent study by Patel et al. (2004) demonstrated a dose-dependent biphasic effect of CP-55,940 on corticosterone secretion induced by restraint stress: Whereas a low dose (0.03 mg/kg CP-55,940) decreased stress-induced corticosterone secretion, a higher dose (0.3 mg/kg) increased stress-induced corticosterone secretion.

Only a few studies have tried to pinpoint down the brain structures involved in the effects of CB1 receptor agonists on HPA axis activity. So far, a consensus has been reached only about a central

Table 2. Survey of articles published on consequences of CB1 receptor agonists for HPA axis activity (chronological order)

Subjects	Drug	Administration	Dose	Time of day	Stressor	ACTH	Corticosterone[a]	References
Male albino rats (Holtzman strain)	Δ^9-THC	i.p. (60 min prior to sacrifice)	5, 10, 20 mg/kg	/	Injection	↑ (determined indirectly via adrenal ascorbic acid content)	n.d.	Dewey et al. (1970)
Healthy human volunteers	Δ^1-THC	p.o. (240 min before blood sampling)	15, 30, 45 mg	08:00–12:00 h	None	n.d.	↔	Hollister et al. (1970)
Male albino rats	Δ^9-THC	i.p. (45 min, 1.5 h, 8 h prior to sacrifice)	2, 4, 8, 16 mg/kg	During light phase (lights on 06:00 h)	Injection	n.d.	↑	Kubena et al. (1971)
Male Sprague–Dawley rats	Δ^9-THC	i.p. (30, 60, 120 min before sacrifice)	2, 5, 10, 20 mg/kg	/	Injection	n.d.	↑	Kokka and Garcia (1974)
Male ICR mice	Δ^9-THC	s.c. (30 min prior to sacrifice)	10, 30, 100 mg/kg (biphasic)	/	Injection	n.d.	↑	Johnson et al. (1978)
	Cannabinol (CBN)		30, 100, 200 mg/kg (biphasic)				↑	
	11-OH-Δ^9-THC		3, 10, 30 mg/kg				↑	
Male albino rats (Camm Wistar)	Δ^9-THC	i.p. (60 min prior to sacrifice)	5 mg/kg	/	Injection	n.d.	↔	Jacobs et al. (1979)
	Cannabidiol (CBD)						↓	
	Δ^9-THC	i.p. (60 min prior to sacrifice; 45 min prior to stress)			Foot shock (1 min)		↑	
	CBD						↔	
Male mice	Δ^9-THC	p.o. (90 min prior to sacrifice)	50 mg/kg	Middle of light cycle	Oral gavage	n.d.	↔	Dalterio et al. (1981)
	CBN						↔	
	Δ^9-THC	p.o. (100 min prior to sacrifice; 90 min prior to stress)			Ether exposure for 30 s; sacrifice 10 min later		↔	
	CBN						↔	
Male rats	Δ^9-THC	i.p. (45 min prior to sacrifice)	5 mg/kg	09:00–10:00 h (lights on 06:00 h)	Injection	↑	↑	Puder et al. (1982)
Male Wistar rats	Δ^9-THC	i.p. (45 min prior to sacrifice)	2, 4 mg/kg	13:00–14:30 h (lights on 06:00 h)	Injection	n.d.	↑	Zuardi et al. (1984)
	CBD		30, 60 mg/kg			n.d.	↑	
Male Sprague–Dawley rats	Δ^9-THC	p.o. (60 min prior to sacrifice)	5 mg/kg	09:00–10:00 h (lights on 08:00 h)	Oral gavage	↔	↑	De Fonseca et al. (1991)
Rats	Δ^9-THC	p.o. (60 min prior to sacrifice)	5 mg/kg	/	Oral gavage	n.d.	↑	Eldridge et al. (1991)
Male rats	Δ^9-THC	i.c.v. (90 min prior to sacrifice)	50, 150 µg/rat	07:00–11:00 h	Injection	↑	↑	Weidenfeld et al. (1994)
	Anandamide					↑	↑	
Wistar rats	HU-210	i.p. (90 min prior to sacrifice)	0.02 mg/kg	/	Injection	↔	↑	De Fonseca et al. (1995)
Male Wistar rats	HU-210	i.p. (60 min prior to sacrifice)	0.02, 0.1 mg/kg	09:30–12:00 h (lights on 08:00 h)	Injection	n.d.	↑	De Fonseca et al. (1996)
Ovariectomized female Sprague–Dawley rats (Harlan)	Δ^9-THC	i.v. via jugular vein catheter (blood sampling 20, 40, 60, 80 min after injection)	0.5, 1 mg/kg	/	Handling	↑	n.d.	Jackson and Murphy (1997)
Male Wistar rats	HU-210	i.p. (3 h prior to sacrifice)	0.1 mg/kg	/	Injection	n.d.	↑	De Fonseca et al. (1997)
Male Sprague–Dawley rats	Anandamide	i.p. (45, 90, 180 min prior to sacrifice)	0.02 mg/kg	/	Injection	n.d.	↑	Wenger et al. (1997)

Table 2. (Continued)

Subjects	Drug	Administration	Dose	Time of day	Stressor	ACTH	Corticosterone[a]	References
Male Wistar rats (Panlab)	HU-210	i.p. (60 min prior to sacrifice)	0.004, 0.02, 0.1 mg/kg	/	Injection	↑	↑	Martin-Calderon et al. (1998)
Male Sprague–Dawley rats	Δ^9-THC	i.c.v. (30 min prior to sacrifice)	25, 50, 100 μg/rat	09:00–12:00 h (lights on 08:00 h)	Injection	↑	↑	Manzanares et al. (1999)
Castrated male calves	WIN 55,212-2 Anandamide/Met-anandamide	i.v. (repeated sampling for up to 2 h after treatment)	2.5, 5 mg/calf 10, 15 mg/calf	/	None	n.d. n.d.	↑ ↑	Zenor et al. (1999)
Male and female Wistar albino rats (Harlan)	CP-55,940	i.p. (40 min prior to sacrifice; 30 min prior to stress)	0.6 mg/kg	09:30–14:30 h (lights on 20:00 h; inverted light dark cycle)	Tail immersion test + holeboard for 5 min	n.d.	↑	Romero et al. (2002)
Male CB1 receptor wt and ko mice (Ledent)	Anandamide	i.p. (90 min prior to sacrifice)	0.01 mg/kg	10:00–15:00 h (lights on 07:00 h)	Injection	wt ↑; ko ↑	wt ↑; ko ↑	Wenger et al. (2003)
Female BALB/cByJ mice (Jackson)	Δ^9-THC	i.v. (3, 5, 10, 18 h prior to sacrifice)	8 mg/kg	/	Injection	n.d.	↑	Newton et al. (2004)
Male Lister-Hooded rats (Charles River)	HU-210	i.p. (45 min prior to sacrifice; 30 min prior to stress)	0.02, 0.08 mg/kg	9:30–16:00 h (lights on 07:00 h)	Open field (15 min) with ultrasound (3 min)	n.d.	↑	Finn et al. (2004a)
Healthy human volunteers	Δ^9-THC	i.v. (repeated sampling 10, 80 and 200 min after treatment)	2.5, 5 mg/subject	/	None	n.d.	↑	D'Souza et al. (2004)
Male ICR mice (Harlan)	CP-55,940	i.p. (60 min prior to sacrifice; 30 min prior to stress)	0.03 mg/kg 0.3 mg/kg	08:00–10:00 h (lights on at 06:00 h)	Restraint (30 min)	n.d.	↓ ↑	Patel et al. (2004)
Schizophrenic patients	Δ^9-THC	i.v. (repeated sampling 10, 80 and 200 min after treatment)	2.5, 5 mg/subject	/	None	n.d.	↑	D'Souza et al. (2005)
Male and female Wistar albino rats (Harlan)	CP-55,940	i.p. (40 min prior to sacrifice; 30 min prior to stress)	0.001, 0.1 mg/kg	09:00–10:00 h (lights on 20:00 h; inverted light dark cycle)	Holeboard (5 min) + elevated plus maze (5 min)	n.d.	↑	Marco et al. (2006)
Male Sprague–Dawley rats (Harlan)	HU-210	i.p. (2.5 and 4.5 h prior to sacrifice) i.p. (30 min before stress; 2.5 and 4.5 h before sacrifice)	0.1 mg/kg	08:30–15:00 h (lights on 08:00 h)	Injection LPS injection (0.1 mg/kg)	n.d. n.d.	↑ ↔	Roche et al. (2006)
Male CD rats (Charles River)	Δ^9-THC	i.p. (15, 30, 60, 90 min prior to sacrifice)	5 mg/kg	/	Injection	↑	↑	Schramm-Sapyta et al. (2007)

Note: i.c.v., intracerebroventricular; i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous; p.o., per os; /, no information provided; n.d., not determined; ↓ decreased secretion, ↔ no change, ↑ increased secretion.
[a]Cortisol in case of human subjects.

role of the PVN. As mentioned before, it has been proposed that the HPA axis stimulating actions of CB1 receptor agonists are centrally mediated (Kubena et al., 1971; Puder et al., 1982), ultimately resulting in the activation of the parvocellular neurons of the PVN. Their activation was demonstrated by CB1 receptor agonist-induced increases in Fos immunoreactivity and in CRH mRNA upregulation (Weidenfeld et al., 1994; Wenger et al., 1997; Corchero et al., 1999). Accordingly, CB1 receptor agonists stimulated the release of CRH from the median eminence into the hypophyseal portal blood (Weidenfeld et al., 1994). Noteworthy, one study has failed to block the stimulating effects of HU-210 on corticosterone secretion by pre-treatment with the CRH antagonist d-Phe CRF_{12-41} (De Fonseca et al., 1996). However, this experiment has to be repeated with a proper dose–response relationship, before this negative finding may falsify the hypothesis that cannabinoids affect HPA axis activity by regulating CRH secretion.

So far it is unknown, which of the extrahypothalamic brain structures are involved in cannabinoid-mediated activation of the PVN. To our knowledge, only one study has yet investigated HPA axis activity in response to brain region-specific injections of CB1 receptor agonists (Finn et al., 2004a; compare the following text below). Up to now one can only speculate from studies examining Fos immunoreactivity. Several different groups could identify a strong Fos activation in the very same brain nuclei following treatment with different CB1 receptor agonists, such as CP-55,940 (Arnold et al., 2001), HU-210 (De Fonseca et al., 1997) and anandamide (Patel et al., 1998). Strikingly, many of those brain areas are strongly implicated in HPA axis regulation, such as the PVN of the hypothalamus, the BNST and the central nucleus of the amygdala (CeA). Interestingly, also the paraventricular nucleus of the thalamus (De Fonseca et al., 1997; Patel et al., 1998; Arnold et al., 2001), and its projection areas, the nucleus accumbens shell (De Fonseca et al., 1997; Arnold et al., 2001) and the prefrontal cortex (Arnold et al., 2001) were strongly activated. The paraventricular nucleus of the thalamus has been specifically implicated in the regulation of autonomic and visceral functions in response to stress, as well as in the integration of HPA axis responses to repeated stressors (Bhatnagar and Dallman, 1998; Herman et al., 2003). In accordance to the Fos findings, which imply an involvement of dopaminergic pathways, De Fonseca et al. (1995) also observed that the stimulatory effects of CB1 receptor agonists on corticosterone secretion can be modulated by chronic pre-treatment with dopamine D1 and D2 receptor agonists, which, however, turned out to control the endocannabinoid system by themselves (Giuffrida et al., 1999; Van der Stelt and Di Marzo, 2003).

The question, whether chronic administration of CB1 receptor agonists leads to tolerance to their stimulatory effects on HPA axis activity could not yet be definitively resolved. Three earlier studies failed to observe tolerance of corticosterone secretion after daily injections of either 8 mg/kg THC for 8 days (Kubena et al., 1971) or even 20 mg/kg THC for 20 days (Kokka and Garcia, 1974), and tolerance of ascorbic acid depletion of the adrenal as an index of ACTH release after 5 days of daily injection with 5 mg/kg THC (Dewey et al., 1970). Two other more recent studies reported clear development of tolerance to the corticosterone stimulating effect of THC after 7 and 14 days of oral treatment with 5 mg/kg THC (De Fonseca et al., 1991; Eldridge et al., 1991). The later studies are supported by findings of Corchero et al. (1999), who showed that repeated injections of 5 mg/kg THC induced tolerance to the stimulating effects of THC on expression of CRH in the PVN (Corchero et al., 1999).

In summary, CB1 receptor agonists have convincingly been demonstrated to activate the HPA axis. However, these findings are not very informative if it comes to the role of endocannabinoids in HPA axis regulation, since exogenous cannabinoids cause a ubiquitous activation of CB1 receptors in an indiscriminative manner, which does not resemble stressor-dependent spatial and temporal signatures of endocannabinoid signalling. Therefore, the next chapter provides an overview over studies, which have used recently established genetic and pharmacological tools (cf. Table 1) in order to elucidate the role of endocannabinoid-mediated CB1 receptor signalling for HPA axis activity.

The role of endocannabinoid signalling for HPA axis function

Table 3 summarizes our current knowledge about the effects of pharmacological or genetic inactivation of CB1 receptors on ACTH and corticosterone secretion. After several negative findings (De Fonseca et al., 1997; Navarro et al., 1997; Wenger et al., 1997), Manzanares et al. (1999) had been the first who reported that blocking CB1 receptors by SR141716 resulted in the very same net effect on HPA axis activation as administration of the exogenous cannabinoid THC. Thus, it was not before 1999 that scientists began to recognize that the endocannabinoid system itself may be a modulator of HPA axis activity, though in the opposite direction to exogenous cannabinoids. It took another 5 years until Patel et al. (2004) brought forward the first thorough investigation on the role of endocannabinoid-mediated CB1 receptor signalling for HPA axis regulation, demonstrating that endocannabinoids generally function to constrain corticosterone release. The findings that administration of a CB1 antagonist leads to an increase in corticosterone secretion could later be verified and extended by Wade et al. (2006) and by our laboratory (Steiner et al., 2008; and Fig. 1 present review). Thus, it became evident that not only CB1 receptor agonists, but also the CB1 receptor antagonist SR141716 stimulates corticosterone secretion in rodents. Similar to THC, also the effect of SR141716 (i) depends on the dose (Manzanares et al., 1999; Patel et al., 2004; Wade et al., 2006; Steiner et al., 2008), is independent (ii) of the sex (Steiner et al., 2008), (iii) of the species (present in both rats and mice) and (iv) of the route of administration. Moreover, the effects are (v) long-lasting (at least 2 h in mice; Steiner et al., 2008; cf. Table 3). As stimulation of corticosterone secretion is prevalent not only after i.p. injection (cf. Table 3), but also after intracerebroventricular injection (Manzanares et al., 1999), the stimulatory effect of SR141716 is supposedly centrally mediated, similar to that of CB1 receptor agonists. However, additional effects on peripheral endocrine glands such as pituitary and adrenals cannot be entirely ruled out.

Dose–response studies that were conducted in mice revealed different effective doses of intraperitoneally administered SR141716, namely 0.3 mg/kg (Wade et al., 2006), 1 mg/kg (Patel et al., 2004) and 2 mg/kg (Steiner et al., 2008). Two studies noted a further increase of the effect size of SR141716 with increasing doses, 5 mg/kg in ICR mice (Patel et al., 2004), and 10 mg/kg in C57BL/6N mice (Steiner et al., 2008), whereas one study already achieved the maximal plateau effect with 0.3 mg/kg SR141716 in C57BL/6 mice, which could not be further elevated with higher doses up to 10 mg/kg (Wade et al., 2006). The reasons for these discrepancies in dose–response relationships are not clear, but may include differences in activation of the endocannabinoid system and sensitivity to the drugs due to differences in the genetic background. In fact, 1 mg/kg SR141716 failed to affect corticosterone secretion in CB1 receptor wild-type mice on a CD1 background, whereas the same dose stimulated corticosterone secretion in C57BL/6 (Wade et al., 2006) and ICR mice (Patel et al., 2004). Wade et al. (2006) have recently studied the effects of different doses of SR141716 on corticosterone secretion in catheterized rats. They observed an activation of the HPA axis with the highest dose tested, 1 mg/kg, 60 min after injection, suggesting that SR141716 has similar HPA axis modulating effects in rats as in mice. However, to our knowledge no dose–response study has been performed employing i.p. injections in rats. This may explain, why some authors have failed to observe a corticosterone-elevating effect of low doses of SR141716 or a structurally similar antagonist, AM251, given i.p. [(1 mg/kg; Wenger et al., 1997; Hill et al., 2006a), (3 mg/kg; De Fonseca et al., 1997; Navarro et al., 1997)], whereas another study showed a strong effect with a dose of 10 mg/kg (Gonzalez et al., 2004; cf. Table 3). Clearly, systematic dose–response studies in rats are warranted.

Some findings in the past have raised doubts about the specificity of SR141716 for CB1 receptors (Haller et al., 2002; Carrier et al., 2005). However, Wade et al. (2006) and our laboratory (Steiner et al., 2008) could demonstrate that the stimulatory effects of SR14716 on corticosterone secretion are likely mediated by CB1 receptors, because SR141716 had no effect in two different

Table 3. Survey of articles published on the role of the endocannabinoid system for HPA axis activity as assessed by genetic or pharmacological inactivation of CB1 receptors (chronological order; but see main text for effects of pharmacological enhancement of endocannabinoid signaling)

Subjects	Interference	Treatment	Dose	Time of day	Stressor	ACTH	Corticosterone	References
Male Wistar rats	SR141716	i.p. (3 h prior to sacrifice)	3 mg/kg	/	Injection	n.d.	↔	De Fonseca et al. (1997)
Male Wistar rats	SR141716	i.p. (60 min prior to sacrifice)	3 mg/kg	09:30–13:00 h (lights on 08:00 h)	Injection	↔	↔	Navarro et al. (1997)
Male Sprague–Dawley rats	SR141716	i.p. (75, 120, 210 min prior to sacrifice)	1 mg/kg	/	Injection	↔	↔	Wenger et al. (1997)
Male Sprague–Dawley rats	SR141716	i.c.v. (60 min prior to sacrifice)	12.5 and 50 μg/rat	09:00-12:00 h (lights on 08:00 h)	Injection	↑	↑	Manzanares et al. (1999)
Male CB1 receptor wt and ko mice (Ledent)	Knockout	i.p. (45, 90, 120, 180 min prior to sacrifice)		10:00–15:00 h (lights on 07:00 h)	Injection	wt Veh–ko Veh ↔;	wt Veh–ko Veh ↔;	Wenger et al. (2003)
	Knockout + SR141716	i.p. (90 min prior to sacrifice)	1 mg/kg			wt Veh–wt SR ↔; ko Veh–ko SR ↔; wt Veh–ko Veh ↔; wt SR–ko SR ↔	wt Veh–wt SR ↔; ko Veh–ko SR ↔; wt Veh–ko Veh ↔; wt SR–ko SR ↔	
Male CB1 receptor wt and ko mice (Ledent)	Knockout			10:00–13:00 h (lights on 06:00 h)	None Novelty (novel empty cage for 10 min; sacrifice immediately after stress)	↑ ↑	↑ ↑	Barna et al. (2004)
Male Lister-Hooded rats	SR141716	i.p. (45 min prior to sacrifice; 30 min prior to stress)	3 mg/kg	09:30–16:00 h (lights on 07:00 h)	Novelty/noise (open field for 15 min with 3 min of ultrasound)	n.d.	↑	Finn et al. (2004a)
Male Wistar rats	SR141716	i.p. (10 min prior to stress; 20 min prior to sacrifice)	10 mg/kg	/	Open field (10 min)	n.d.	↑	Gonzalez et al. (2004)
Male CB1 receptor wt and ko mice (Ledent)	Knockout			/	None Novelty (novel empty cage for 10 min)	↑ ↑	n.d. n.d.	Haller et al. (2004)
Male ICR mice (Harlan)	SR141716	i.p. (60 min prior to sacrifice)	1 and 5 mg/kg	08:00–10:00 h (lights on 06:00 h)	Injection	n.d.	↑	Patel et al. (2004)
		i.p. (30 min prior to stress; 60 min prior to sacrifice)			Restraint (30 min)	n.d.	↑	
Male CB1 receptor wt and ko mice (Ledent)	Knockout			09:00-13:00 h (lights on 07:00 h)	None	n.d.	↓	Urigüen et al. (2004)

Table 3. (Continued)

Subjects	Interference	Treatment	Dose	Time of day	Stressor	ACTH	Corticosterone	References
Male CB1 receptor wt and ko mice (Zimmer)	Knockout			10:00–12:00 h	None	↔	↔	Fride et al. (2005)
					Bell noise (4 min; sacrifice 10 min after stressor onset)	↔	↔	
Male Sprague–Dawley rats	AM251	i.p. (110 min prior to sacrifice)	1 mg/kg	/	Injection	n.d.	↔	Hill et al. (2006a)
		i.p. (60 min prior to stress; 110 min prior to sacrifice)	1 mg/kg	/	Forced swim (5 min)	n.d.	↔	
Male Sprague–Dawley rats (Harlan)	SR141716	i.p. (30 min prior to stress; 2.5 and 4.5 h prior to sacrifice)	3 mg/kg	08:30–15:00 h (lights on 08:00 h)	Injection of LPS (0.1 mg/kg)	n.d.	↑	Roche et al. (2006)
Male C57BL/6 mice (Taconic)	SR141716	i.p. (60 min prior to sacrifice)	0.3, 1, 3, 10 mg/kg	/	Injection	n.d.	↑	Wade et al. (2006)
Male Wistar rats	SR141716	i.v. via jugular vein catheter (60 min prior to sacrifice)	1 mg/kg	/	Injection	n.d.	↑	
Male CB1 receptor wt and ko mice (Zimmer)	Knockout			/	None	n.d.	↔	
Male CB1 receptor wt and ko mice (Zimmer)	Knockout + SR141716	i.p. (60 min prior to sacrifice)	1 mg/kg	/	Injection	n.d.	wt Veh−wt SR ↑; ko Veh−ko SR ↔; wt Veh−ko Veh ↔; ko SR−wt SR ↑	
Male rough-skinned newts (amphibians)	AM281	i.c.v.	0.5 and 5 μg/newt	/	Injection	n.d.	↔	Coddington et al. (2007)
					Rotation stress	n.d.	↔	
Female CB1 receptor wt and ko mice (Marsicano)	Knockout			05:30–06:30 h (lights on 06:00 h)	None	↔	↔	Cota et al. (2007)
				17:30–18:30 h (lights on 06:00 h)	None	↔	↑	
		i.p. (6 h prior to sacrifice)		17:30–18:30 h (lights on 06:00 h)	Injection (vehicle)	↑	↑	

Male CB1 receptor wt and ko mice (Ledent)	Knockout			/	None	n.d.	↔	Aso et al. (2008)
					Tail suspension (6 min; sacrifice 1 h later)	n.d.	↑	
Male C57BL/6N mice (Charles River)	SR141716	i.p. (60 min prior to sacrifice)	2, 10 mg/kg	01:00–07:00 h (lights on 07:00 h)	Injection	n.d.	↑	Steiner et al. (2008)
		i.p. (60 min prior to stress; 70 min prior to sacrifice)	10 mg/kg		Forced swim (6 min)	n.d.	↑	
Male and female CB1 receptor wt and ko mice (Marsicano)	Knockout			15:00–21:00 h (lights on 21:00 h; inverted light dark cycle)	None	n.d.	Males ↑; Females ↔	
					Forced swim (6 min; sacrifice 30 min after stressor onset)	n.d.	↑	
	Knockout + SR141716	i.p. (60 min prior to stress; 70 min prior to sacrifice)	10 mg/kg	15:00–21:00 h (lights on 21:00 h; inverted light dark cycle)	Forced swim stress (6 min)	n.d.	wt Veh−wt SR ↑; ko Veh−ko SR ↔; wt Veh−ko Veh ↑; wt SR−ko SR ↔	
Male C57BL/6J mice (Charles River)	SR141716	i.p. (70 min prior to sacrifice)	3 mg/kg	09:00–11:00 h (lights on 07:00 h)	Injection	n.d.	↑	Steiner and Wotjak (present review)
		i.p. (60 min prior to stress; 70 min prior to sacrifice)			Social defeat (10 min)	n.d.	↑	
Male CB1 receptor wt and ko mice (Marsicano)	Knockout			05:30–06:30 h (lights on 06:00 h)	None	n.d.	↔	
				17:30–18:30 h (lights on 06:00 h)	None	n.d.	↑	

Note: i.c.v., intracerebroventricular; i.p., intraperitoneal; i.v., intravenous; /, no information provided; n.d., not determined; ↓ decreased secretion, ↔ no change, ↑ increased secretion.

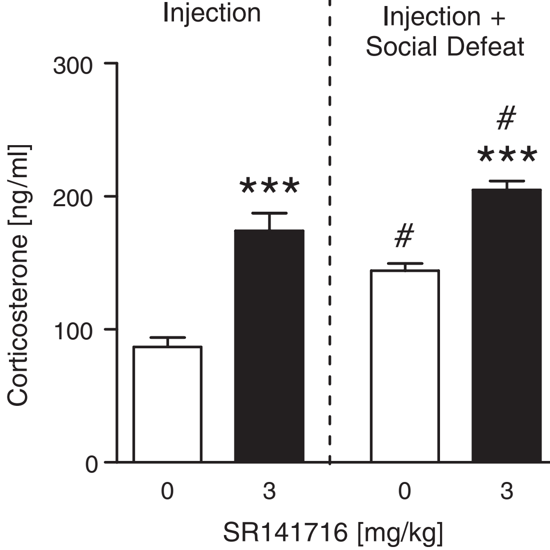

Fig. 1. Acute pharmacological blockade of CB1 receptors increases injection stress- and social defeat stress-induced corticosterone secretion. Male C57BL/6J mice were acutely treated with vehicle (i.p. injection) or SR141716 (3 mg/kg; i.p.). Seventy minutes after injection, half of the animals were killed for blood sampling without further stressor exposure. Sixty minutes after injection, the other half was exposed to social defeat stress for 10 min according to a previously established protocol (Berton et al., 2006) and killed directly after stressor termination. Animal experiments were approved by the UT Southwestern's Institutional Animal Care and Use Committee (TX, USA). The experiment was conducted between 9:00 and 11:00 am (lights on at 7:00 am). The pharmacological blockade of CB1 receptors by SR141716 led to an increase in injection- and social defeat stress-induced corticosterone secretion [treatment: $F_{1,28} = 72.1$, $p < 0.001$; 2-way ANOVA (stress, treatment)]. As compared to injection stress levels, social defeat stress itself led to a general increase in corticosterone secretion in all groups (stress: $F_{1,28} = 25.4$, $p < 0.001$), independent of treatment (treatment × stress: $F_{1,28} = 2.3$, $p = 0.14$). $n = 8$ mice per group; $^{***}p < 0.001$ vs. respective vehicle group; $^{\#}p < 0.05$ vs. respective injection stress control group; Newman-Keuls Post-hoc analysis.

CB1 receptor knockout strains, but in the respective wild-type littermate controls.

Another similarity to the effects of CB1 receptor agonists on HPA axis activity is the fact that SR141716 is not only able to increase mild injection-stress-induced corticosterone secretion, but also to further increase the corticosterone response elicited by more severe stressors, such as exposure to ultrasound and an open field (Finn et al., 2004a), to the forced swim test (Steiner et al., 2008) or to restraint stress (Patel et al., 2004). Moreover, the effect seems to apply to both, physical stressors, such as lipopolysaccharide injection (Roche et al., 2006), and to stressors with a strong psychological component, such as exposure to an open field (Gonzalez et al., 2004) or to social defeat (Fig. 1).

With the development of CB1 receptor-deficient mice, not only pharmacological but also genetic tools became available for studying the role of endocannabinoid signalling for HPA axis regulation (Table 3). Independently of the genetic background, CB1 receptor knockout mice showed increased ACTH and corticosterone levels, under basal conditions (Barna et al., 2004; Haller et al., 2004; Cota et al., 2007; Steiner et al., 2008) as well as in response to novelty stress (Barna et al., 2004; Haller et al., 2004), injection stress (Cota et al., 2007), forced swim stress (Steiner et al., 2008) and tail suspension stress (Aso et al., 2008), thus resembling the effect of antagonist treatment (Table 3). There are a few instances, however, where no differences could be observed between CB1 receptor-deficient mice and wild-type controls. For example, Wenger et al. (2003) found no genotype differences 90 min after an i.p. vehicle injection in CB1 receptor mutant mice of the CD1 background (Ledent et al., 1999). This finding is at odds with later studies on the very same mutant line, which could clearly show HPA axis hyperactivity in CB1 receptor knockout mice (Barna et al., 2004; Haller et al., 2004; Aso et al., 2008). Another two studies (Fride et al., 2005; Wade et al., 2006) found no basal, injection stress- or bell stress-induced genotype differences in a different CB1 receptor mutant strain on the C57BL/6J background (Zimmer et al., 1999). As Wade et al. (2006) pointed out, these findings raise the question as to whether CB1 receptor knockout mice on the C57BL/6J background show developmental compensatory changes for the lifelong loss of CB1 receptors. However, findings in CB1 receptor-deficient mice on the related C57BL/6N background (Marsicano et al., 2002) closely resembled the effects of SR141716 on corticosterone secretion following injection and forced swim stress (Steiner et al., 2008). In order to prove that CB1 knockout mice on the C57BL/6J background show, indeed, a

normal HPA axis function, additional studies are required, which include time-course analyses and exposure to different stressors.

The cellular and molecular mechanisms underlying the effect of endocannabinoids on HPA axis regulation have only begun to be deciphered and still offer plenty opportunities for future research. CB1 receptor knockout mice show elevated levels of CRH mRNA in the PVN (Cota et al., 2003, 2007), which indicates a sustained activation of the HPA axis. This observation goes hand in hand with a recently developed electrophysiology model for glucocorticoid-mediated fast-feedback regulation in these neurons, which is based on endocannabinoid signalling (Di et al., 2003). The authors proposed that increased corticosterone levels feed back onto membrane-bound and G-protein-coupled glucocorticoid receptors in PVN neurons. Thereby corticosterone triggers the local synthesis of both anandamide and 2-AG (Di et al., 2005; Malcher-Lopes et al., 2006), which travel retrogradely over the synaptic cleft to bind to CB1 receptors on glutamatergic afferences of the PVN and to result in a reduction in spontaneous glutamate release from these terminals (see Fig. 2, for a more detailed description of this process). If this scenario holds true also in vivo (which still remains to be shown and cannot be taken for granted), this corticosterone-mediated fast-feedback may result in a reduced activation of CRH-containing PVN neurons and, in consequence, in a re-setting of the HPA axis following stressor exposure. Its crucial dependence on the endocannabinoid system would explain the exaggerated hormonal stress responses observed in CB1 receptor-deficient mice and following administration of CB1 receptor antagonists.

Interestingly, rapid feedback mechanisms of corticosterone via membrane-bound receptors seem to represent a common regulatory principle (Dallman, 2003; Tasker et al., 2006; De Kloet et al., 2008; Joels et al., 2008), which could be observed not only in the PVN, but also in magnocellular vasopressinergic and oxytocinergic neurons of the HNS (Di et al., 2005) and within the hippocampus (Karst et al., 2005). However, whereas corticosterone leads to a decrease in glutamatergic transmission within the HNS in a similar manner as observed in parvocellular neurons of the PVN (see Fig. 3 for details), it activates GABAergic transmission within the HNS (Di et al., 2005) and glutamatergic transmission within the hippocampus (Karst et al., 2005). The latter two processes appear to be independent of endocannabinoid signalling, but to involve either nitric oxide signalling (in case of GABAergic transmission within the HNS; Di and Tasker, WCNH 2007, Abstract) or membrane-bound mineralocorticoid rather than glucocorticoid receptors (in case of glutamatergic transmission within the hippocampus; Karst et al., 2005; Joels et al., 2008). It is important to note that dendritic and axonal release of vasopressin and oxytocin, controlled by endocannabinoids or not (Fig. 3), may substantially contribute to HPA axis regulation and behavioural stress coping (Engelmann et al., 1996, 2004).

It seems paradoxical that CB1 receptor antagonists could provide the same net effect on HPA axis function as CB1 receptor agonists. However, the picture is very complex because CB1 receptor signalling controls both, glutamatergic excitatory neurotransmission, and GABAergic inhibitory neurotransmission (compare text further above), which might be differentially affected by CB1 receptor agonists and antagonists. In fact, the PVN receives both direct glutamatergic and GABAergic afferences from surrounding hypothalamic areas as well as indirect innervations from limbic brain regions with at least one trans-synaptic relay (for review see Herman et al., 2004).

There are two promising approaches for investigating the neuronal substrates of cannabinoid- and endocannabinoid-mediated HPA axis regulation, namely brain region-specific infusions of CB1 receptor agonists or antagonists and analysis of mouse mutants with conditional ablation of CB1 receptors. In terms of local drug treatment, Finn et al. (2004a) could already demonstrate that the administration of 5 μg HU-210 into the periaqueductal grey of the rat brain was sufficient to significantly increase ultrasound-evoked corticosterone levels. With respect to the second approach, there is a steadily increasing number of conditional CB1 receptor knockout mice, which lack the CB1 receptor in certain neuronal subpopulations only (e.g., only in GABA- or glutamatergic neurons),

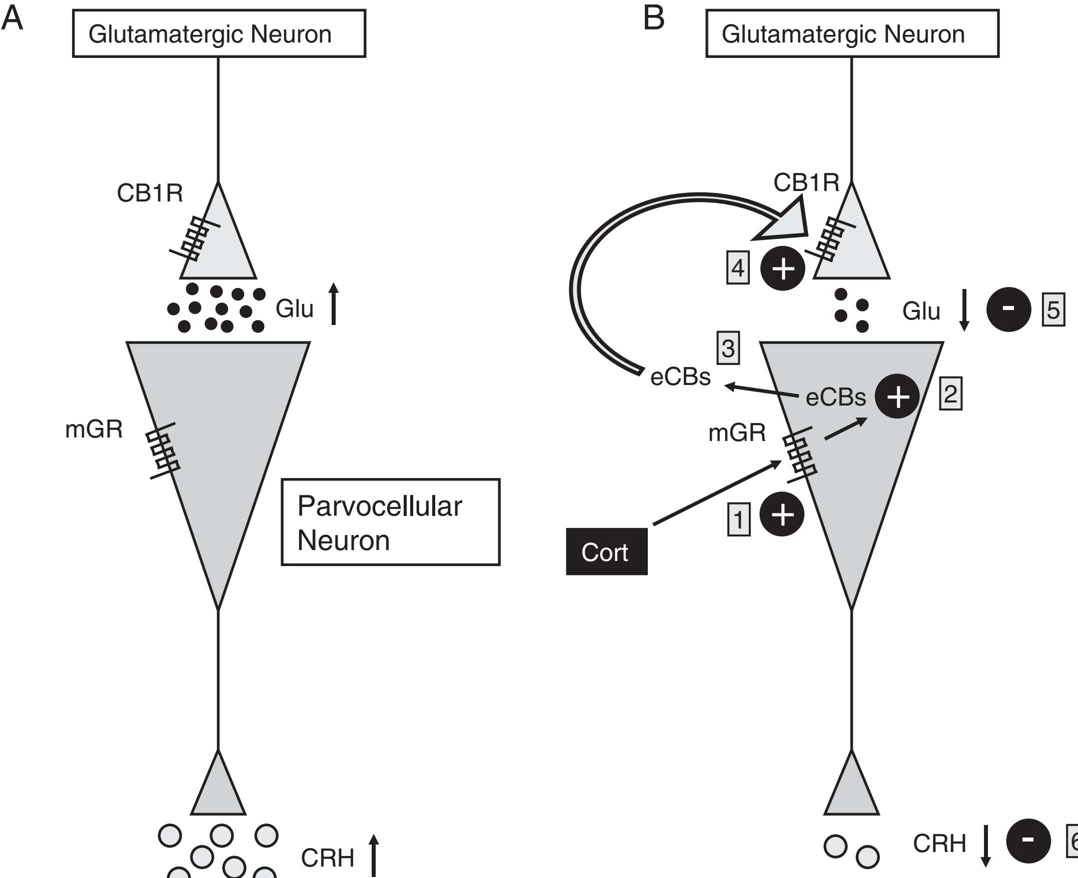

Fig. 2. Involvement of the endocannabinoid system in rapid negative feedback of glucocorticoids on activation of parvocellular neurons of the PVN. (A) During stressful encounters, parvocellular neurons of the PVN become activated by excitatory glutamatergic afferences, thus leading to an increased release of CRH into the portal blood, of ACTH from the anterior pituitary (not shown) and of corticosterone from the adrenals (not shown). (B) Rapid negative feedback of corticosterone at level of the PVN involves endocannabinoid signalling. Binding of corticosterone to membrane-bound glucocorticoid receptors (mGR) leads to an activation of $G\alpha_S$-proteins and, subsequently, of the cAMP pathway (1). As a consequence, endocannabinoids (eCBs; anandamide and 2-AG) are synthesized (2) and released from postsynaptic compartments into the extracellular space (3). Binding to CB1 receptors (CB1R; 4), which are localized on presynaptic terminals of afferent glutamatergic neurons, results in a reduction of glutamate release (5) and, in consequence, of CRH release (6), thus leading to a subsequent dampening of HPA axis activity. This schematic presentation is based on findings obtained under in vitro conditions (Di et al., 2003; Malcher-Lopes et al., 2006). It remains to be shown whether the endocannabinoid-mediated down-regulation of glutamatergic transmission plays a significant role in HPA axis regulation also under in vivo conditions.

or in certain areas of the brain (for currently available mutant strains, see Monory et al., 2006, 2007). It would be interesting to see, how administration of CB1 receptor agonists or antagonists affects HPA axis activity in these mutants.

Another finding, which deserves further investigations, relates to the fact that anandamide may still exert its corticosterone-elevating effects in mice with genetic ablation of CB1 receptors (Wenger et al., 2003). This result suggests that anandamide may act on other targets beside CB1 receptors, such as a yet unknown cannabinoid receptor or the vanilloid receptor TRPV1. The latter option appears to be less likely, because the corticosterone-elevating effect of anandamide could not be blocked by inhibition of TRPV1 receptors with capsazepine (Wenger

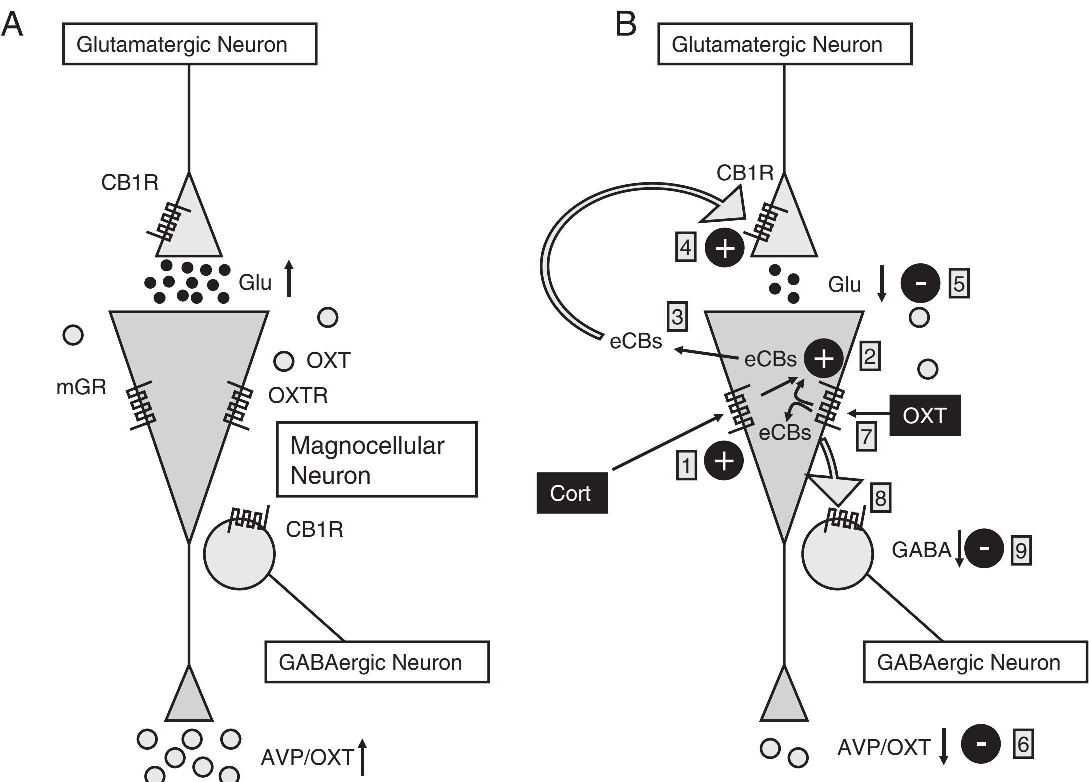

Fig. 3. Involvement of the endocannabinoid system in feedback regulation of magnocellular neurons of the hypothalamus. (A) The activity level of both vasopressinergic and oxytocinergic magnocellular neurons of the hypothalamic PVN and supraoptic nucleus (SON) are controlled by excitatory glutamatergic neurons. (B) Rapid negative feedback of corticosterone at level of the magnocellular neurons involves binding of corticosterone to membrane-bound glucocorticoid receptors (mGR) (1) and activation of endocannabinoid (eCB) synthesis (2) and release (3). Endocannabinoid binding to presynaptically localized CB1 receptors (CB1R; 4) results in a down-regulation of glutamate release (5), thus leading to a subsequent reduction in the release of vasopressin and oxytocin (OXT) from axon terminals (6). This regulatory mechanism has been described for both oxytocinergic and vasopressinergic neurons (Di et al., 2005). In addition, also dendritically released oxytocin may trigger endocannabinoid synthesis and release, thus further promoting inhibition of glutamatergic transmission (Hirasawa et al., 2004). Other than for excitatory transmission, GABAergic transmission becomes activated rather than inhibited by corticosterone, most likely without involvement of the endocannabinoid system (not shown; Di et al., 2005), but with a significant contribution of nitric oxide signalling (Di and Tasker, WCNH 2007, Abstract). Interestingly, also GABAergic transmission might be negatively controlled by endocannabinoids. In this case, however, activation of oxytocin receptors (OXTR) by dendritically released oxytocin (7) leads to a tonic activation of endocannabinoid synthesis and release (8) and, in consequence to a downregulation of GABA release (9) (Oliet et al., 2007). Other than for the restraining of glutamate release, glucocorticoid- or OXT-controlled endocannabinoid signalling at GABAergic synapses is expected to result in increased rather than decreased secretion of the neuropeptide from axon terminals. Moreover, endocannabinoids seem to mediate the inhibitory effects of α-melanocyte-stimulating hormone (α-MSH) on spontaneous activity of oxytocinergic neurons (Sabatier and Leng, 2006; not shown), which may serve as a switch between dendritic and axonal oxytocin release (Sabatier et al., 2003). Taken into account that magnocellular neurons may contribute to HPA axis regulation (Engelmann et al., 2004), with differential contribution of dendritic and axonal neuropeptide release (Wotjak et al., 2002), endocannabinoids may regulate HPA axis activity not only within the PVN (Fig. 2), but also at level of the HNS.

et al., 2003). However, a recent electrophysiological study revealed that the TRPV1 agonist capsaicin caused an increase in excitatory glutamatergic transmission within the PVN (Li et al., 2004). Therefore, it would be worthwhile to investigate HPA axis function in TRPV1 knockout mice (Caterina et al., 2000), in particular since TRPV1 seems to mediate opposite effects on cognition and

emotionality compared to CB1 receptors (Marsch et al., 2007). The nature of yet unknown additional cannabinoid receptors in the brain is still under investigation. However, recent evidence suggests that CB2 receptors might be present in the brain (Van Sickle et al., 2005) and that an orphan GPCR (GPR55; Baker et al., 2006) might be a cannabinoid receptor. At the moment, there are no reliable data available regarding the putative role of brain CB2 and GPR55 in the regulation of the HPA axis.

Also FAAH knockout mice, which show highly elevated anandamide levels (Cravatt et al., 2001), could be investigated with respect to HPA axis regulation. This mouse line may be specifically valuable for dissecting the contribution of anandamide vs. 2-AG for HPA axis regulation. To our knowledge, only one study investigated the consequences of enhanced endocannabinoid signalling on corticosterone secretion so far. Both blockade of anandamide degradation by URB597 and blockade of endocannabinoid re-uptake by AM404 attenuated stress-induced corticosterone secretion in ICR mice (Patel et al., 2004). These dampening effects on HPA axis activity might be very specific for the kind and duration of stressor exposure (30 min restraint), since we failed to detect any effect of URB597 on either injection stress- or FST stress-induced corticosterone secretion (Fig. 4), under conditions, which clearly involve endocannabinoid signalling (Steiner et al., 2008). Certainly, there is a considerable demand for additional studies, which thoroughly investigate HPA axis modulating effects of endocannabinoid enhancing drugs, including dose–response relationships and exposure to different stressors during different times of the day, when the responsiveness of the HPA axis changes considerably (Atkinson et al., 2006; Girotti et al., 2007).

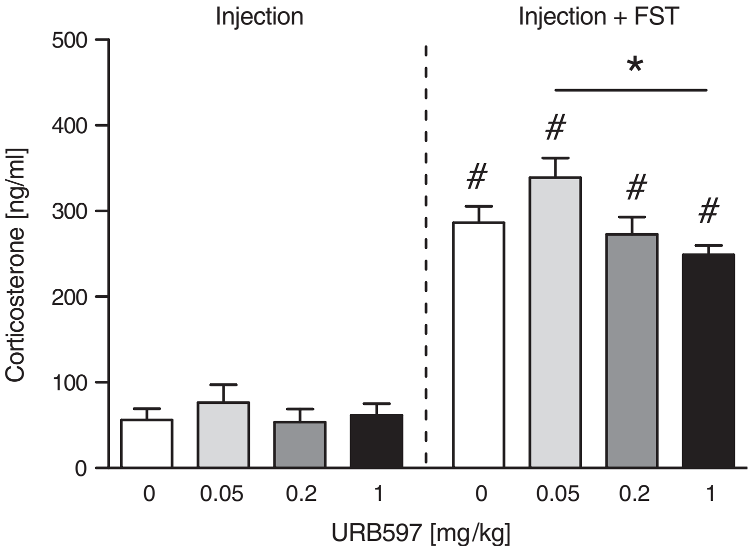

Fig. 4. Effect of acute pharmacological blockade of fatty acid amide hydrolase (FAAH) on injection stress- and forced swim test (FST) stress-induced corticosterone secretion. Male C57BL/6N mice were acutely treated with vehicle (i.p. injection) or the FAAH inhibitor URB597 (0.05, 0.2, 1 mg/kg; i.p.). Seventy minutes after injection, half of the animals were killed for blood sampling without further stressor exposure. Sixty minutes after injection, the other half was exposed to FST stress for 6 min and killed 4 min after stressor termination. Animal experiments were approved by the UT Southwestern's Institutional Animal Care and Use Committee (TX, USA). The experiment was conducted between 1:00 and 4:00 am (lights on at 7:00 am). 2-way ANOVA (stress, treatment) of corticosterone levels showed an overall effect of URB597 (treatment: $F_{3,39} = 3.47$, $p<0.05$). As compared to injection stress levels, FST stress itself led to a general increase in corticosterone secretion in all groups (stress: $F_{1,39} = 319.1$, $p<0.001$), independent of treatment (treatment $\times$ stress: $F_{1,39} = 1.56$, $p = 0.22$). Further one-way ANOVA (treatment) of the FST stress groups revealed a significant effect of URB597 ($F_{3,20} = 4.01$, $p<0.05$), with the 0.05 mg/kg group being significantly different from the 1 mg/kg group, but not the vehicle group, as revealed by Newman–Keuls Post-hoc analysis. $n = 5$–6 mice per group; $^{*}p<0.05$; $^{\#}p<0.001$ vs. respective injection stress control group.

Interestingly, stimulatory effects of endocannabinoids on basal HPA axis activity seem to depend on the circadian time of testing. We could show in female CB1 receptor knockout mice an increase in basal corticosterone secretion at the peak (i.e., around the onset of the dark phase), but not the nadir (i.e., the onset of the light phase) of the circadian rhythm of corticosterone secretion (Cota et al., 2007). These findings could be corroborated by current results investigating basal corticosterone levels in male CB1 receptor knockout mice

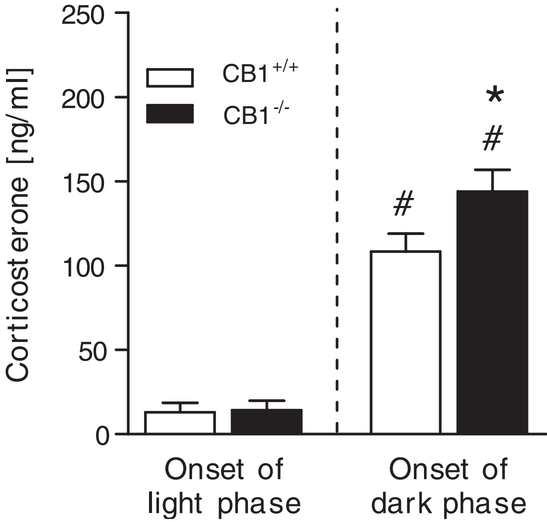

Fig. 5. Circadian changes of basal plasma corticosterone levels of CB1 receptor wild-type ($CB1^{+/+}$) and knockout ($CB1^{-/-}$) mice. Male $CB1^{+/+}$ and $CB1^{-/-}$ littermates were derived from heterozygous breedings and backcrossed to the C57BL/6N background for six generations (Marsicano et al., 2002). Mice were killed for circadian corticosterone measurements at the onset of the light phase (06:00 am) and at the onset of the dark phase (18:00). Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria (Germany). The circadian hormonal variation was preserved in both $CB1^{+/+}$ and $CB1^{-/-}$ mice, as both genotypes showed significantly higher corticosterone levels at the onset of the dark phase than at the onset of the light phase [time: $F_{1,70} = 237.7$, $p<0.001$; 2-way ANOVA (time × genotype)]. Further planned pair-wise comparison revealed increased corticosterone levels of $CB1^{-/-}$ mice when compared to $CB1^{+/+}$ mice only at the onset of the dark phase ($t_{35} = 2.1$, $p<0.05$; Student's t-test). n = 16–21 mice per group; $^{*}p<0.05$ vs. respective $CB1^{+/+}$ group; $^{\#}p<0.001$ vs. respective corticosterone levels at the onset of the light phase as revealed by Newman-Keuls Post-hoc analysis.

(Fig. 5). Interestingly, only male, but not female, CB1 receptor knockout mice showed elevated basal corticosterone levels also towards the end of the dark phase (i.e., activity phase of the animals; Steiner et al., 2008), thus indicating that endocannabinoids may influence basal corticosterone secretion in a sex-dependent and circadian time-dependent manner. The latter observation implies that endocannabinoids restrain corticosterone levels when basal HPA axis activity is at its height, with little impact during the trough. This may explain discrepant findings from CB1 mutant mice on the CD1 background (Ledent et al., 1999), which showed either decreased (Uriguen et al., 2004), increased (Haller et al., 2004) or similar (Aso et al., 2008) corticosterone and/or ACTH levels as compared to wild-type mice (compare also Table 3). No basal changes of corticosterone levels have so far been detected in CB1 receptor mutant mice on the C57BL/6J background (Zimmer et al., 1999; Fride et al., 2005; Wade et al., 2006). To explain the dependency of endocannabinoid action on the circadian rhythm we propose that the concentration of corticosterone has to exceed a certain threshold at level of the PVN, before it may activate local endocannabinoid synthesis and release (Fig. 2).

Endocannabinoid signalling and behavioural stress coping

Reviews on an involvement of the endocannabinoid system in regulation of the HPA axis can hardly go without mentioning potential implications of endocannabinoids in behavioural stress coping. At this place, we will only provide a short survey of the respective literature. Readers who are interested in more detailed information are referred to recent comprehensive review articles on this subject (Carrier et al., 2005; Hill and Gorzalka, 2005a; Viveros et al., 2005; Wotjak, 2005; Witkin et al., 2005a). Behavioural stress coping is typically assessed by stress-induced alterations in analgesia, certain forms of memory, anxiety-like behaviour, sexual behaviour, sensitivity to natural reward and behavioural measures of despair in the context of both acute or chronic

stressor exposure. Most of these processes are affected by endocannabinoids and, to a large extent, also by glucocorticoids. In the following we will discuss potential roles of endocannabinoid-controlled HPA axis activity exemplarily for stress-induced analgesia, extinction of aversive memories, sexual behaviour, behavioural despair and coping with chronic stress. The extensive literature on the role of endocannabinoids and stress on learning and memory and emotionality will be reviewed elsewhere (Kamprath et al., in preparation).

Stress-induced analgesia

Acute stressor exposure, such as application of an inescapable foot shock or forced swimming, results in stress-induced analgesia, which is typically assessed by an increase in latencies of jumping or tail flick responses to heat or a reduction in formalin-evoked nociceptive behaviour. Several studies report a significant role of the endocannabinoid system in non-opioid stress-induced analgesia (Vaughan, 2006), demonstrating reduced analgesia in CB1 deficient mice (Valverde et al., 2000) and in rats with systemic SR141716 treatment (Finn et al., 2004b). More recently, the periaqueductal grey could be identified as the major site of endocannabinoid action for stress-induced analgesia (Hohmann et al., 2005), whereas the role of the basolateral amygdala is less evident (Connell et al., 2006; Roche et al., 2007). Enhancement of endocannabinoid signalling within the periaqueductal grey by pharmacological means was able to potentiate stress-induced analgesia (Hohmann et al., 2005). Despite some positive results (Fukuda et al., 2007), corticosterone secretion seems to be of minor importance, in particular for opioid-dependent stress-induced analgesia (Contet et al., 2006). Therefore, it is unlikely that alterations in HPA axis activity account for the effects of endocannabinoids.

Extinction of aversive memories

Modulation of endocannabinoid signalling (cf. Table 1) was shown to interfere with a variety of learning and memory tasks (for reviews see Wotjak, 2005; Kamprath et al., in preparation). Of importance, in the context of the present review, is the observation that both glucocorticoids (Korte, 2001) and endocannabinoids (Lutz, 2007) are involved in the extinction of aversive memories. It could be shown that activation of endocannabinoid signalling promotes (Chhatwal et al., 2005) and attenuation impairs the extinction of fear memories (Marsicano et al., 2002; Suzuki et al., 2004; Chhatwal et al., 2005; Pamplona et al., 2006; Niyuhire et al., 2007). Corticosterone, in turn, promotes memory extinction in inhibitory avoidance (for review see Korte, 2001) and fear conditioning tasks (Barreto et al., 2006; Cai et al., 2006; Yang et al., 2006). The fact that endocannabinoids restrain corticosterone secretion (Table 3), but promote fear extinction similarly to corticosterone renders it unlikely that alterations in HPA axis activity are involved in endocannabinoid effects on memory extinction, which, however, remains to be shown.

Sexual behaviour

A recent publication provides a very striking example of how behavioural effects of corticosterone depends on endocannabinoid signalling (Coddington et al., 2007; see also the comment by Denver, 2007). The authors could demonstrate that clasping of female rough-skinned newts (*Taricha granulosa*) by male conspecifics is impaired, if the males have undergone rotation stress or have received an i.p. injection of corticosterone before exposure to the female. In both cases, the attenuation of clasping behaviour could be reversed by intracerebroventricular administration of the CB1 receptor antagonist AM281. The effects of the antagonist were not mediated secondarily via modifications of the HPA axis (which remained unaffected by AM281), but restored the activity of medullary neurons, which had been suppressed by corticosterone-mediated increased endocannabinoid signaling. The disruption of clasping behaviour and its dependence on endocannabinoid signaling provides one of the best examples for the physiological significance of rapid non-genomic effects of glucocorticoids that are mediated via membrane-bound corticosteroid receptors (Orchinik et al.,

1991). This mechanism is reminiscent of the rapid action of corticosterone on parvocellular (Fig. 2) and magnocellular neurons (Fig. 3) of the hypothalamus of rats.

Behavioural despair

A variety of studies measured consequences of altered endocannabinoid signalling on behavioural despair in the forced swim or the tail suspension test. In most cases the authors reported a decrease in immobility time (Tzavara et al., 2003; Shearman et al., 2003; Griebel et al., 2005; Steiner et al., 2008) following genetic deletion or pharmacological blockade of CB1 or no effect (Gobbi et al., 2005; Gobshtis et al., 2007). The behavioural phenotype of CB1 receptor-deficient mice appears to be less prevailing (Shearman et al., 2003; Jardinaud et al., 2005; Steiner et al., 2007, 2008), and CB1 receptor-deficient mice occasionally showed even an increase in immobility time in the forced swim (Steiner et al., 2007) and the tail suspension test (Aso et al., 2008). It is conceivable that compensatory processes due to the lifelong absence of CB1 receptors account for the discrepant findings obtained after genetic and pharmacological inactivation of CB1 receptors. Yet, the alteration in stress-coping behaviour observed in CB1 receptor-deficient mice coincided with a decrease in the expression of brain-derived neurotrophic factor (BDNF) within the hippocampus (Aso et al., 2008; Steiner et al., 2007); and restoration of BDNF signalling recovered the phenotype of the mutants (Aso et al., 2008). This is an interesting observation, since BDNF has been suggested as a key player for the development of psychiatric disorders including major depression (Duman and Monteggia, 2006).

Surprisingly, not only blockade, but also potentiation of endocannabinoid signalling by pharmacological or genetic means leads to a decrease in immobility times (Gobbi et al., 2005; Hill and Gorzalka, 2005b; Hill et al., 2007b; Naidu et al., 2007) with a significant impact of the testing conditions (Naidu et al., 2007). Both, increased endocannabinoid signalling and exogenously applied CB1 agonists seem to promote active stress coping by increasing the firing activity of serotonergic neurons within the dorsal raphe nucleus (Gobbi et al., 2005; Bambico et al., 2007). Evidence exists that this process may involve the neuronal subpopulation, which projects from the prefrontal cortex to the dorsal raphe nucleus (Bambico et al., 2007) and which has been implicated in inhibiting serotonergic pathways during controllable stress (Amat et al., 2005). Also activation of CB1 receptors within the dentate gyrus of the dorsal hippocampus promote active stress coping behaviour, as revealed by local administration of the CB1 receptor agonist HU-210 (McLaughlin et al., 2007). The fact, however, that neither pharmacological blockade of local CB1 receptors by AM251 nor blocking of local anandamide degradation by URB597 affected forced swimming behaviour (McLaughlin et al., 2007) argues against a significant involvement of the endocannabinoid system within the dentate gyrus in the development of behavioural despair.

Corticosterone has been reported to promote immobility behaviour in the forced swim test (for review see Korte, 2001). However, at least in mice it appears to be unlikely that corticosterone secretion plays an important role for the behavioural phenotype observed in this task, since mice with completely blunted HPA axis, due to genetic deletion of the CRH receptor type 1, behaved normally in the forced swim test (Sillaber et al., 2002; Lu et al., submitted). Also the reduced level of glucocorticoid receptors observed in the hippocampus of CB1 deficient mice (Cota et al., 2007), which would be expected to result in a decrease in behavioural despair (for review see Urani et al., 2005), did not coincide with a reduction in immobility time in the very same CB1 receptor knockout mice (Steiner et al., 2008).

Chronic stress

Another group of studies investigated the involvement of the endocannabinoid system in behavioural adaptation to repeated stressor exposure. In ICR mice, habituation of the HPA axis response to repeated restraint stress (Patel et al., 2004) coincided with a decrease in anandamide levels within the amygdala but an increase in 2-AG levels within the forebrain, the amygdala and the

hypothalamus as assessed immediately at the end of the last 30-min restraint episode (Patel et al., 2004, 2005). Fos staining revealed that blockade of CB1 receptors by SR141716 before the fifth restraint episode reversed habituation to the homotypic stressor in the neuronal circuitry, thus indicating that endocannabinoids might actively suppress stress-induced activation of the lateral septum, the nucleus accumbens and various areas of the prefrontal cortex after repeated restraint stress (Patel et al., 2005). In addition, mice showed more escape behaviour, if CB1 receptors were blocked before the first or the fifth restraint episode (Patel et al., 2005). An independent study, which employed the same mouse strain and a similar experimental protocol, largely confirmed the consequences of repeated restraint stress on habituation of the HPA axis and endocannabinoid levels, with anandamide levels showing primarily a reduction and 2-AG levels primarily an increase in a brain structure-dependent manner with increasing number of stressor exposures (Rademacher et al., 2008). In the same animals, the activity of the endocannabinoid degrading enzymes FAAH and MGL was increased in the prefrontal cortex, whereas CB1 receptor binding parameters remained unaltered within amygdala, ventral striatum and prefrontal cortex (Rademacher et al., 2008). A similar repeated stressor exposure caused a decrease in sucrose consumption, which is indicative of increased anhedonia, a key symptom of animal models of depression. Co-administration of a CB1 receptor agonist (CP-55,940) or a FAAH inhibitor (URB597) partially blocked restraint-stress-induced anhedonia, whereas blockade of CB1 receptors by SR141716 promoted it without affecting sucrose consumption in non-restraint controls (Rademacher and Hillard, 2007). Pronounced anhedonia could also be observed in CB1 receptor-deficient mice subdued to a chronic unpredictable mild stress protocol (Martin et al., 2002). Conversely, chronic treatment with URB597 partially reversed adverse effects of chronic unpredictable mild stress on body weight gain and sucrose intake as well as the stress-induced increase in CB1 receptor mRNA levels within the prefrontal cortex of Wistar rats (Bortolato et al., 2007). This time, chronic stress failed to significantly affect endocannabinoid levels except for a slight increase in 2-AG levels within the thalamus, assessed 2 h after a last vehicle injection. However, the failure to detect significant changes in hippocampal CB1 mRNA in chronically stressed Wistar rats (Bortolato et al., 2007) is at odds with the decreased CB1 protein levels and receptor binding seen in the hippocampus of similarly chronically stressed Long-Evans rats (assessed 12 h after initiation of a last 12-h overnight social isolation; Hill et al., 2005). The latter changes in hippocampal CB1 levels were accompanied by increased perseverance of spatial memory upon reversal learning in the water maze (Hill et al., 2005), an effect, which is reminiscent of the behavioural phenotype of CB1-deficient mice (Varvel and Lichtman, 2002) and which could be reversed by treatment with the CB1 receptor agonist HU-210 (Hill et al., 2005). The beneficial effects of HU-210 treatment on cognition, however, have to be paid for by an increased sensitivity of the animals to anxiogenic-like consequences of the drug (Hill and Gorzalka, 2004). Interestingly, not only chronic unpredictable stress increases the sensitivity of the animals to CB1 agonists, but, vice versa, chronic treatment with HU-210 also leads to sensitization of the hormonal stress response to an acute restraint stress (Hill and Gorzalka, 2006). The effects of chronic stress on hippocampal CB1 receptor levels (Hill et al., 2005) could be mimicked by chronic, but not acute, treatment with corticosterone (Hill et al., 2007a), suggesting a significant role of glucocorticoids in long-lasting alterations of the endocannabinoid system.

Taken together the heterogeneous set of data, endocannabinoids seem to mediate certain aspects of stress-induced analgesia and stress-induced attenuation of sexual behaviour in amphibians. Furthermore, they affect acute behavioural coping in the forced swim and the tail suspension test. They also seem to play a distinct role in the extinction of aversive memories and in habituation to homotypic stressors (i.e. repeated restraint stress), a phenomenon, which may, at least partially, account also for the impairments in fear extinction observed in CB1 receptor-deficient mice (Kamprath et al., 2006). Similar to the shared effects of CB1 receptor antagonists and agonists

on HPA axis activity, both inhibition and promotion of endocannabinoid signalling occasionally have the same consequences on behavioural stress coping in the forced swim or the tail suspension test because of yet unknown reasons. So far, there is no direct evidence for a causal relationship between the effects of endocannabinoids on HPA axis regulation and their effects on behavioural stress coping, except for the disturbances of sexual behaviour in male rough-skinned newts (Coddington et al., 2007).

In terms of chronic unpredictable mild stress, the majority of the studies consistently reported that blockade of endocannabinoid signaling promotes the development and/or the expression of depression-like phenotypes of the animals, whereas activation of the endocannabinoid system exerts opposite effects. Studies on the activity of the endocannabinoid system, in contrast, revealed less consistent findings in terms of stress-induced alterations in endocannabinoid and CB1 receptor levels. These data, however, have to be interpreted with caution, because of the aforementioned limitations of these approaches (see text above). Also the involvement of endocannabinoids in adult neurogenesis within the dentate gyrus (Aguado et al., 2005; Jin et al., 2004; Jiang et al., 2005; Hill et al., 2006b; for review see Galve-Roperh et al., 2007) deserves further investigations in terms of behavioural consequences of chronic stress and/or chronically elevated corticosterone level, in particular if one considers that several authors have implemented reduced neurogenesis in the aetiology of depressive symptoms (Duman, 2004; Sahay and Hen, 2007; but also see Henn and Vollmayr, 2004).

Noteworthy, one study demonstrated that blockade of CB1 by SR141716 has beneficial effects in OF1 mice in terms of amelioration of stress-induced degradation in physical state and reversal of stress-induced anxiety and behavioural despair following chronic unpredictable mild stress (Griebel et al., 2005). These discrepancies including antidepressant-like effects of CB1 receptor blockade in the forced swim test illustrate the complexity of the involvement of the endocannabinoid system in stress coping. Moreover, they might explain the controversies about the potential impact of this system and its pharmacological exploitation in context of psychiatric disorders such as depression. Whereas some authors suggested a therapeutic benefit of a pharmacological blockade of CB1 (Witkin et al., 2005b), others proposed that blockade of endocannabinoid signalling represents a novel animal model of melancholic depression (Hill and Gorzalka, 2005a). To resolve this issue, the following chapter deals with the putative involvement of the endocannabinoid system in aetiology and therapy of human depression.

Endocannabinoid system and melancholic depression

It has been Hill and Gorzalka (2005a), who very convincingly outlined in an excellent review article the similarities between the behavioural responses of CB1 receptor-deficient mice and symptoms of melancholic depression in terms of depressed mood, anxiety, anhedonia, reduced food intake, pronounced weight loss, predominance of aversive emotional memories, hippocampal atrophy and hyperactivity of the HPA axis. The latter endophenotype deserves particular attention in the context of our article. HPA axis dysfunctions seem to play a pivotal role in aetiology, therapy resistance and incomplete remission of severe depressive episodes, and their reversal allows predictions about the success of antidepressant treatment (Holsboer, 2001; De Kloet et al., 2005). Notwithstanding melancholic depression is primarily not a neuroendocrine disorder, chronically elevated cortisol levels and an impaired negative feedback seem to affect a variety of endophenotypes of major depression (De Kloet et al., 2005; Hill and Gorzalka, 2005a). In light of these arguments, animal experiments reporting HPA axis hyperactivity (Steiner et al., 2008) or increased stress-induced anhedonia following chronic pharmacological blockade of CB1 (Rademacher and Hillard, 2007) suggest that both phenotypes might interfere with each other, thus causing a depression-like phenotype in the animals. Strikingly enough, meta-analyses of four major double-blind placebo-controlled clinical studies on rimonabant

(i.e. the CB1 antagonist SR141716; Table 1) in obesity (RIO; Despres et al., 2005; Van Gaal et al., 2005; Pi-Sunyer et al., 2006; Scheen et al., 2006) revealed that the relative risk for psychiatric adverse events in subjects chronically treated with 20 mg SR141716 was 1.9 as compared to placebo, with 9% vs. 5% of the participants reporting symptoms of depression and 6% vs. 1.5% reporting symptoms of anxiety. Subjective experience of stress, in contrast, was not different after treatment with verum or placebo. Survival analyses revealed that the psychiatric adverse events developed early in treatment [Food and Drug Administration Endocrinologic and Metabolic Advisory. June 13, 2007. Briefing Information, NDA 21-888 ZIMULTI (rimonabant) — Sanofi-Aventis. 2007. http://www.fda.gov/OHRMS/DOCKETS/AC/07/briefing/2007-4306b1-00-index.htm; assessed Aug. 6, 2007].

On the basis of the same RIO studies, Christensen et al. (2007) independently came to similar conclusions as the FDA. In addition, they made the important remark that the population of patients participating in the RIO studies were highly selected, since patients with a past history of severe depression or those with present severe psychiatric illness were excluded and antidepressant treatment was not permitted and warranted mandatory treatment discontinuation. Hence, one can assume that the treatment with SR141716 might even have more adverse consequences in patients with a history in melancholic depression and/or anxiety disorders. Unfortunately, there are no reports about effects of SR141716 on HPA axis activity in human subjects [at least the stimulatory effects of exogenous cannabinoids seen in animal experiments seem to be preserved in humans (compare Table 2)]. It is tempting to speculate that rimonabant treatment has caused hyperactivity of the HPA axis, which could have contributed to the aetiology of the mood disorders in the susceptible population of the patients, even more if one considers that chronically elevated corticosterone level may lead to a down-regulation of CB1 receptors in certain brain regions (Hill et al., 2007a).

In addition to the well-controlled RIO studies, which, in total, included approximately 1600 patients treated with placebo, 2500 patients treated with 5 mg SR141716 and 2500 patients treated with 20 mg SR141716, there are also other reports about alterations in the endocannabinoid system in depressed patients. For instance, one study demonstrated an upregulation of CB1 binding and activity within the prefrontal cortex of depressed suicide victims by means of radioligand binding, Western blot and [^{35}S]GTPγS binding (Hungund et al., 2004), which is reminiscent of the increase in CB1 mRNA in the prefrontal cortex of chronically stressed rats (Bortolato et al., 2007). Other authors reported a reduced prevalence of depression in Parkinson's disease, if patients bear more than 16 triplet repeats in the *CNR1* gene coding for the human CB1 receptor (Barrero et al., 2005). However, since association studies failed to reveal a relationship between triplet repeat polymorphisms in the promoter region of the *CNR1* gene and the pathogenesis or the psychotic symptoms of mood disorder (Tsai et al., 2001), such polymorphisms might play a role in a selected subset of depressed patients only.

Interestingly, a potential interrelation between the endocannabinoid system and the incidence of depression is supported by epidemiological studies on extensive cannabis consumption (for reviews see Johns, 2001; Raphael et al., 2005), demonstrating an association between increasing cannabis use and a two- to threefold increase in self-reported levels of depression (Rey and Tennant, 2002; Rey et al., 2002), a fourfold increase in the risk for major depression with a specific association in suicidal ideation and anhedonia (Bovasso, 2001) and a two- to fivefold increase in the risk for later depression and anxiety in case of weekly or more frequent cannabis use in teenage girls (Patton et al., 2002). Importantly, neither baseline depression nor baseline anxiety predicted later cannabis use, thus ruling out that cannabis consumption coincided with a priori mood disturbances simply because of its application for self-medication, rather than being causative for an increase in the prevalence for anxiety disorders and depression (Patton et al., 2002). It is important to note, however, that clinical and epidemiological studies are not always controlled for treatment history, in particular with antidepressants. Interactions between the endocannabinoid

system and antidepressants have been observed in several preclinical studies (Oliva et al., 2005; Hill et al., 2006a; Steiner et al., 2007, 2008) and deserve further investigations both in humans and animals.

The fact that both CB1 antagonists and exogenous agonists seem to promote the development of depression is surprising and reminiscent of antagonist and agonist effects on HPA axis activity in animal studies (Tables 2 and 3). It is currently unknown which cellular processes account for these similarities. They likely involve effects on different neuronal populations (Monory et al., 2007) and seem to depend on the doses applied (Carrier et al., 2005). In addition, both acute and sustained activation of CB1 receptors may cause metaplastic changes in the endocannabinoid system, which are characterized, among others, by reduced CB1 binding and signalling (McKinney et al., 2008) and alterations in interneuronal communication, including synaptic plasticity (Mato et al., 2004; Hoffman et al., 2007). This would explain why chronic cannabis consumption and pharmacological blockade of CB1 by means of rimonabant have the same adverse consequences on mood and anxiety, which likely involve dysregulations of the HPA axis.

Conclusion

Animal experiments performed over the course of the past 10 years identified the endocannabinoid system of the brain as an important homoeostatic principle, which constrains HPA axis activity under basal and stress conditions and counteracts overshooting behavioural stress responses. It is likely that endocannabinoids exert their effects on ACTH and corticosterone secretion at different levels of the HPA axis. Of particular importance might be their involvement in rapid negative feedback actions of corticosterone at level of the hypothalamus, which includes down-regulation of excitatory transmission within the PVN. This striking interdependence between corticosterone levels and endocannabinoid signalling might not only play a role during stress, but also explain the specific involvement of endocannabinoids in regulation of HPA axis activity during the peak of the circadian rhythm of corticosterone secretion. Evidence exists that chronic activation of the HPA axis, as seen during chronic stress or following chronic corticosterone administration, leads to plastic changes in endocannabinoid synthesis and expression of CB1 receptors, which attenuates the protective effects of the endocannabinoid system on the development of depression-like symptoms. Accordingly, amplification of endocannabinoid signalling at their site of action by blocking endocannabinoid uptake and degradation ameliorated the deleterious consequences of chronic stress. The protective effects of endocannabinoids gain particular importance in light of large-scale human studies on anti-obesity effects of the CB1 receptor antagonist rimonabant (i.e. SR141716), which revealed a higher incidence of depression and anxiety symptoms. Strikingly enough, exogenous CB1 receptor agonists, such as, Δ^9-THC, show similar stimulatory effects on HPA axis activity as CB1 receptor antagonists. The reasons for these similarities are still unknown. The observation, however, that excessive marijuana consumptions coincides with a higher incidence of depression and anxiety, similar to rimonabant, favors a scenario, according to which high concentrations of CB1 agonist as well as long-lasting activation of corticosterone secretion leads to desensitization of CB1 receptors and, in consequence, to an attenuation of the protective effects of endocannabinoid signalling. The exact mechanisms, however, how endocannabinoids and exogenous cannabinoids regulate HPA axis activity and mood are far from being clear and deserve major research efforts in the future.

Acknowledgements

This work was partly supported by grants from the National Institute of Mental Health and by a PhD grant of the Boehringer Ingelheim Foundation to M.A. Steiner. We would like to thank Eric J. Nestler (Department of Psychiatry, The University of Texas Southwestern Medical Center, Dallas, TX, USA) for his generous support concerning the experiments leading to Figs. 1 and 4 of the present review. We would like to thank Giovanni Marsicano (INSERM Bordeaux, France) and Beat Lutz

(University of Mainz, Germany) for their generosity in providing the CB1 mutant mice and for their comments on this manuscript.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 34

Molecular genetic studies of the arginine vasopressin 1a receptor (*AVPR1a*) and the oxytocin receptor (*OXTR*) in human behaviour: from autism to altruism with some notes in between

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Abstract: Converging evidence from both human and animal studies has highlighted the pervasive role of two neuropeptides, oxytocin (OXT) and arginine vasopressin (AVP), in mammalian social behaviours. Recent molecular genetic studies of the human arginine vasopressin 1a (*AVPR1a*) and oxytocin (*OXTR*) receptors have strengthened the evidence regarding the role of these two neuropeptides in a range of normal and pathological behaviours. Significant association between both *AVPR1a* repeat regions and *OXTR* single nucleotide polymorphisms (SNPs) with risk for autism has been provisionally shown which was mediated by socialization skills in our study. *AVPR1a* has also been linked to eating behaviour in both clinical and non-clinical groups, perhaps reflecting the social and ritualistic side of eating behaviour. Evidence also suggests that repeat variations in *AVPR1a* are associated with two other social domains in *Homo sapiens*: music and altruism. *AVPR1a* was associated with dance and musical cognition which we theorize as reflecting the ancient role of this hormone in social interactions executed by vocalization, ritual movement and dyadic (mother-offspring) and group communication. Finally, we have shown that individual differences in allocation of funds in the dictator game, a laboratory game of pure altruism, is predicted by length of the *AVPR1a* RS3 promoter-region repeat echoing the mechanism of this hormone's action in the vole model of affiliative behaviours and facilitation of positive group interactions. While still in its infancy, the current outlook for molecular genetic investigations of AVP-OXT continues to be fascinating. Future studies should profitably focus on pharmacogenomic and genomic imaging strategies facilitated by the ease and efficacy of manipulating AVP-OXT neurotransmission by intranasal

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DOI: 10.1016/S0079-6123(08)00434-2

administration. Importantly, physiological measures, behavioural paradigms and brain activation can be informed by considering between-group and also within-group individual differences defined by common polymorphisms. Ultimately, investigators should strive to develop a cohesive model explaining how genomic variations are translated into individual and group differences in higher-order social behaviours.

Keywords: arginine vasopressin 1a receptor (*AVPR1a*); oxytocin receptor (*OXTR*); molecular genetics; social behaviour; autism; neuropeptides; music

Introduction

Emerging research on the molecular genetic foundations of arginine vasopressin (AVP) and oxytocin (OXT) mediation of human behaviour has been the fortuitous beneficiary of three proximal research streams: (1) The well-documented role of OXT and AVP receptor genes (*AVPR1a*, *OXTR*) in mammalian behaviour, particularly the vole (Young, 1999), has stamped the molecular genetic signature of these two neuropeptides on affiliative behaviours across species and kindled interest in finding similar mechanisms in their human homologues; (2) association studies of both *AVPR1a* (Kim et al., 2002; Wassink et al., 2004; Yirmiya et al., 2006) and *OXTR* (Wu et al., 2005; Jacob et al., 2007; Lerer et al., 2007) examine their role in autism, a disorder whose core deficits are centred on social interaction and communication. These clinical investigations by studying an extreme phenotype have also underscored the potential importance of these two neuropeptides in normal human social behaviours and (3) a new bag of tools has been provided by the burgeoning field of behavioural neuroeconomics (Adolphs, 2003; Camerer, 2007), emphasizing novel laboratory-based paradigms to assess the neurocognitive architecture of social behaviour. Of particular interest are studies showing that administration of intranasal administration of OXT caused subjects to display a remarkable change in social behaviour and related brain activity, including modulated amygdala responses to facial expressions, a willingness to trust anonymous partners in an economic task and an improved ability to recognize emotions (Kirsch et al., 2005; Kosfeld et al., 2005; Domes et al., 2007a, b). While these studies have helped clear the waters for a general understanding of how OXT and AVP function as social facilitators, the basis for the often significant individual differences in these phenotypes has been left unexplored, providing a unique opportunity for molecular geneticists.

Behavioural effects of AVP and OXT are species-specific with the neuropeptides exerting their actions via binding to specific receptors. Both OXT (Gimpl and Fahrenholz, 2001) and AVP receptors (Thibonnier et al., 2002) have seven transmembrane domains and belong to the class of G-protein-coupled receptors. In humans, at least three vasopressin receptor types (V_1R/V1a, V_2R and V_3R/V1b) have been identified (Thibonnier et al., 2002; Streefkerk and van Zwieten, 2006). Of special relevance for human behavioural studies is *AVPR1a* (chr 12q14-15) because regional brain-expression patterns of the V1a receptor gene determine marked intra- and interspecies variation in social and reproductive behaviour in the vole model (Hammock and Young, 2002, 2004, 2005, 2006; Hammock et al., 2005). Therefore, research in human social behaviour has perforce focused on the *AVPR1a* receptor. However, the $V_{1b}R$ (V_3R) receptor type is also of interest because some studies have linked it to anxiety and depression (Ring, 2005).

AVPR1a

The species-specific pattern of *AVPR1a* brain expression is determined by repeat elements (microsatellites) in the upstream receptor promoter region (Hammock and Young, 2002, 2004, 2005, 2006; Hammock et al., 2005). In a remarkable series of investigations, the longer length of the repeat in the prairie vole was shown to explain its gregarious nature and affiliative behaviours. Individual

differences in prairie vole behaviours are also explained by within-species microsatellite variation that determines gene expression (Hammock and Young, 2005). Intriguingly, our research group also showed that upstream promoter-region microsatellite length appears to modulate a spectrum of *human* social behaviours including some that resonate with similar phenotypes observed in lower mammals (Bachner-Melman et al., 2004, 2005a, c, 2007; Knafo et al., 2008).

Located on chromosome 12q14-15, *AVPR1a* is distinguished by three microsatellites in the 5′ flanking region and a fourth in the single intron (Thibonnier et al., 2000; Thibonnier, 2004). Following the vole studies, attention has been primarily focused on two microsatellites in the promoter region, RS1 and RS3, which are highly polymorphic (many repeat alleles of varied lengths are represented in the population) and which have been the mainspring of research to date; see Fig. 1 for a schematic. Most importantly, as shown in Fig. 2, the first findings using human postmortem hippocampal samples showed that long *AVPR1a*. RS3 repeats (>325 bp) were associated with higher *AVPR1a* mRNA levels than short repeats, revealing a functional molecular genetic basis for the differences in social behaviour (Knafo et al., 2008) and as discussed later on in this chapter.

OXTR

The human *OXTR* is a 389-amino acid polypeptide located on chromosome 3p25 spanning approximately 19 kbp and containing three introns and four exons (Inoue et al., 1994). Approximately 30 single nucleotide polymorphisms (SNPs) are known in the *OXTR* gene region. An intelligent SNP marker selection scheme has been developed which is aimed at increasing the chances that at least one typed SNP would be in linkage disequilibrium (LD) with the disease/phenotype causative variant, while at the same time controlling the cost of the study in terms of the number of markers genotyped and samples. Empirical studies reporting block-like segments in the genome with high LD and low haplotype diversity have motivated a marker selection strategy whereby subsets of SNPs that 'tag' the common haplotypes (htSNPs) of a region are selected for genotyping, avoiding typing redundant SNPs (Halldorsson et al., 2004).

Following this strategy of cost-effective and efficient SNP selection, we used HapMap data http://www.hapmap.org/ and the Haploview programme (Barrett et al., 2005) to identify 18 tagging htSNPs across the *OXTR* gene region. As discussed below, we genotyped these tagging SNPs and tested association with autism (Lerer et al., 2007). The location of these htSNPs along the

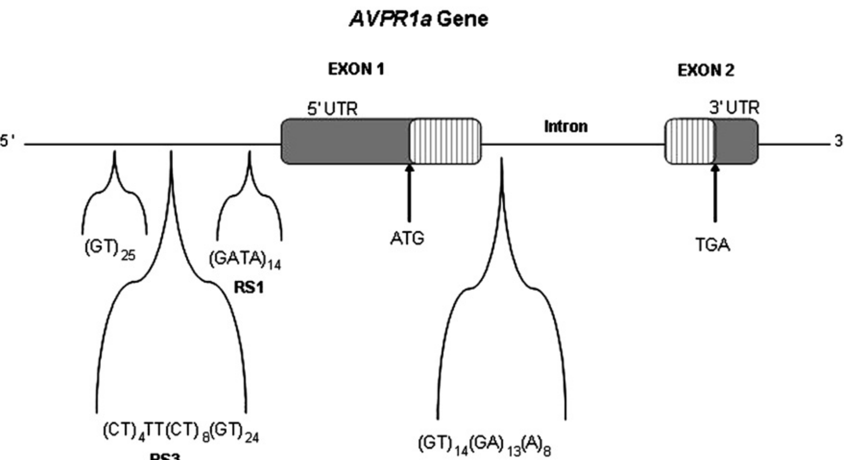

Fig. 1. Location of *AVPR1a* microsatellite repeats (Thibonnier et al., 2000). The start site of protein synthesis is represented by ATG.

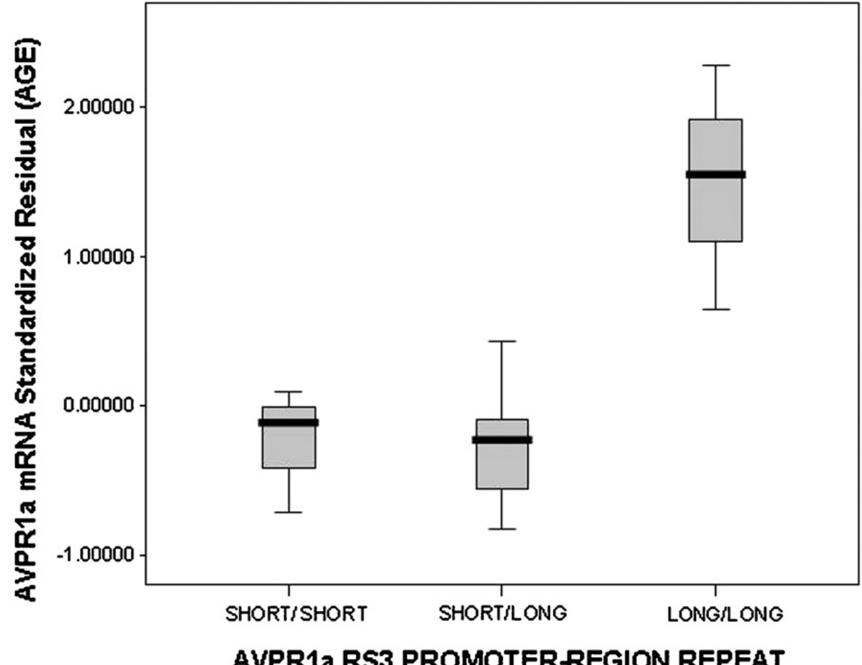

Fig. 2. Hippocampal *AVPR1a* mRNA levels grouped by *AVPR1a* RS3 promoter-region repeat by genotype length. The data is presented as a SPSS box plot. Control subjects had no contact with any psychiatric service prior to death, had not received anti-psychotic medication, had not died by suicide nor had any neurological disorder. Age: 46.6 years ± 15.2 (S.D.); sex: 4F/11M, post-mortem interval (PMI): 42.0 h ± 16.4 (S.D.) and pH of brain tissue: 6.3 ± 0.22 (S.D.). Post-mortem mRNA levels correlated only with age. We also analysed the data using the non-parametric Kruskal Wallis statistic (chi-square = 6.8, 2 DF, $p = 0.033$).

OXTR genomic region and their relationship to the exon/intron boundaries are displayed in Fig. 3.

Genetic studies

Clinical studies: autism

Mediation of social behaviours by AVP and OXT in animal models led a number of investigators to postulate that variants in these two neuropeptides could potentially contribute risk to human behavioural disorders, especially autism spectrum disorders (ASD) (Insel et al., 1999; Lim et al., 2005; Carter, 2007; Hammock and Young, 2006). ASD, which is no longer uncommon (Newschaffer et al., 2007), is a pervasive neuropsychiatric disorder marked by a triad of impairments including deficits in social interaction and communication, and repetitive and restrictive behaviours and interests.

Kim et al. (2002) first demonstrated an association between *AVPR1a*.RS3 microsatellites and autism. Two later studies by Wassink et al. (Wassink et al., 2004) and our group (Yirmiya et al., 2006) provided independent replications of this first finding. Wassink et al. (2004) found modest evidence for LD in both the RS1 and the RS3. We (Yirmiya et al., 2006) demonstrated that the association between autism and *AVPR1a* is partly mediated by deficits in socialization skills. The demonstration that the risk conferred by *AVPR1a* for autism is partially mediated by socialization skills strengthens the link between the well-characterized role of this social hormone in other mammals and humans. Moreover, these results suggest the somewhat remarkable notion that during the long course of vertebrate evolution including *Homo sapiens*, these two neuropeptides have a conserved role in enabling social and affiliative behaviours.

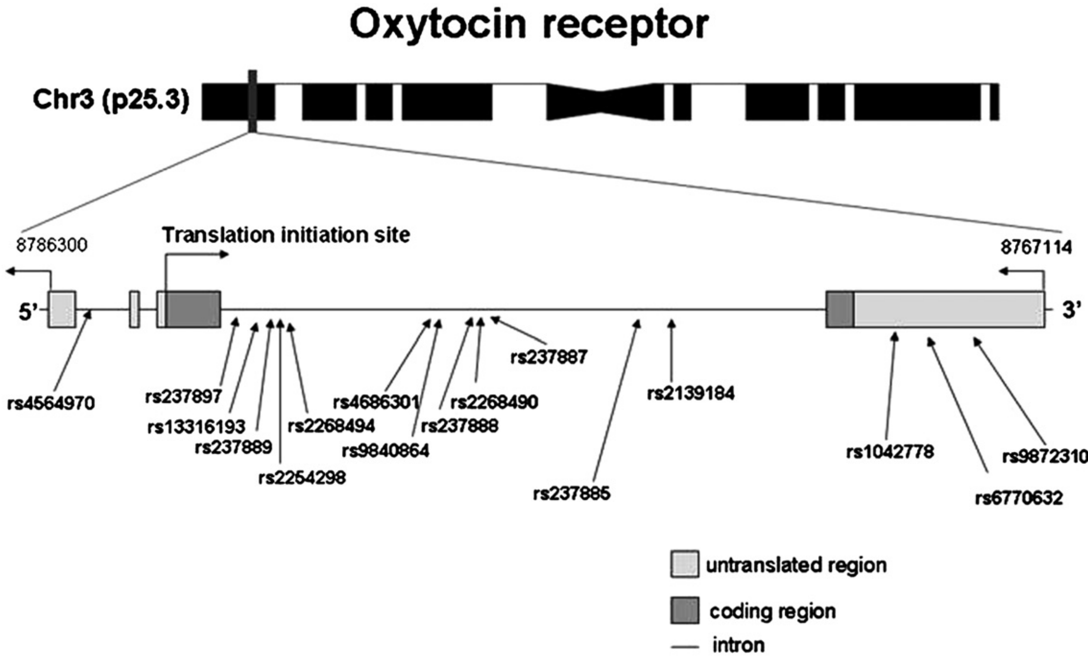

Fig. 3. Position of tagged SNPs sites and their relationship to the exon–intron boundaries of the *OXTR* gene.

Recently, Wu et al. (2005) genotyped four SNPs in *OXTR* in 195 Chinese Han trios and demonstrated association with ASD with both individual SNPs and haplotypes. A second study by Jacob et al. (2007) genotyped two *OXTR* SNPs and observed a significant association with a single SNP (rs2254298) in Caucasian children and adolescents with autism. We undertook a comprehensive study of all 18 tagged SNPs across the entire *OXTR* gene region (Fig. 3). Altogether 152 subjects diagnosed with autism spectrum disorders (ASD, i.e. DSM IV autistic disorder or pervasive developmental disorder — NOS) from 133 families were genotyped (parents and affected siblings). Both individual SNPs and haplotypes were tested for association using family-based association tests as provided in the UNPHASED set of programmes (Koeleman et al., 2000; Dudbridge, 2003). Significant association with single SNPs and haplotypes (global p values <0.05, following permutation test adjustment) were observed with ASD. Association was also observed with IQ and the Vineland adaptive behaviour scales (VABS) scores. In particular, a five locus haplotype block (rs237897-rs13316193-rs237889-rs2254298-rs2268494) was significantly associated with ASD (nominal global $p = 0.000019$; adjusted global $p = 0.009$), and a single haplotype (carried by 7% of the population) within that block showed highly significant association ($p = 0.00005$).

Our study was the third association study, in a third ethnic group, revealing that SNPs and haplotypes in the *OXTR* gene confer risk for ASD. We also showed association with IQ and total VABS scores (as well as the communication, daily living skills and socialization subdomain scores), suggesting that this gene shapes both cognition and daily living skills which may have a role in other disorders, as well as non-clinical populations.

A role for the *OXTR* in autism is further strengthened by two genome-wide scans highlighting the 3p25 region, containing the *OXTR* receptor gene, as a putative linkage site for ASD (McCauley et al., 2005; Lauritsen et al., 2006). Finally, a unique case study that lends additional credence to the role of *OXTR* in autism is the

description of a 9-year-old boy referred for genetic testing and presented with pervasive developmental disorder, delayed speech and rapid onset obesity. Extensive genetic analysis revealed an apparent duplication of chromosome region 3p25.3p26.2 and a twofold–threefold increase of *OXTR* expression relative to comparison subjects (Bittel et al., 2006).

Other clinical studies

Investigations of other clinical groups extended the evidence about the influence of AVP receptors in a broader set of phenotypes, still linked albeit indirectly, to social behaviour. For example, *AVPR1a* microsatellites have been associated with anorexia nervosa and child and adolescent perfectionism (Bachner-Melman et al., 2007) as well as with general eating pathology in a non-clinical population (Bachner-Melman et al., 2004); behaviours that we hypothesize are inextricably entrenched in social settings and rituals, in culinary and consumption customs and in cultural habits and norms. Geller et al. (2005) explored an association between *AVPR1a* and child hypersexuality in 32 bipolar families, finding no significant associations between RS1 or RS3 repeats and uninhibited behaviour. In one of the rare reported studies examining the 1b receptor, van West et al. (2004) genotyped five SNPs in both Belgian and Swedish populations, and found a significant protective effect of an *AVPR1b* haplotype on major depression.

AVPR1a and social behaviour in non-clinical populations

Interpersonal relationships: siblings and others

We (Bachner-Melman et al., 2005c) examined RS1 and RS3 promoter-region repeat markers and tested for linkage to two complex social behaviours in humans: sibling relationships and self-presentation style. We evaluated the perceived quality of the relationship between siblings using the sibling relationship questionnaire (SRQ; Furman and Buhrmester, 1985) and assessed three dimensions including: (1) relative status/power, (2) warmth/closeness and (3) conflict. Self-presentation style was assessed by employing the concern for appropriateness scale (CFA), which measures a defensive and fearful social approach associated with conformity and aimed at gaining acceptance and approval, and avoiding social threats (Lennox and Wolfe, 1984). The CFA reflects social orientations with a high degree of concern for social cues and social approval and correlates negatively with self-esteem and extraversion. One might expect the prairie vole to score high on both the SRQ (e.g. warmth/closeness) and the CFA scale.

These two self-report questionnaires were administered to 552 same-sex siblings from 248 families. Suggestive linkage was observed between both microsatellites (RS1 and RS3) and the SRQ conflict scale (RS1: $\chi^2 = 13.65$, LOD $= 2.96$, $p = 0.0001$; RS3: $\chi^2 = 14.54$, LOD $= 3.16$, $p = 0.00007$) and the CFA scale self-presentational style (RS1: $\chi^2 = 8.25$, LOD $= 1.79$, $p = 0.002$; RS3: $\chi^2 = 8.81$, LOD $= 1.91$, $p = 0.002$). These results provided the first provisional evidence that the *AVPR1a* polymorphism predicts normal social behaviour in humans and linked a specific genetic element to perceived sibling interactions. It is tempting to speculate that sibling relationships and the phenotypes represented by the CFA scale are human representations of some of the behaviours observed in other mammals that are also partially mediated by the *AVPR1a* receptor.

Music

Dance

As evidenced by several prominent reviews (Koelsch and Siebel, 2005; Zatorre and McGill, 2005), there is increasing interest in the neurobiological substrates of musical ability and appreciation. Additionally, various theories of the 'why and how' of musical evolution have been suggested including its importance in mother–infant communication, sexual selection and group cohesion (see discussion in Fitch, 2005, 2006).

In a first study of its kind, we recruited 85 current performing dancers and their parents who were genotyped for the serotonin transporter (*SLC6A4*: promoter-region HTTLPR and intron

2 VNTR) and *AVPR1a*: promoter microsatellites RS1 and RS3 (Bachner-Melman et al., 2005a). We also genotyped 91 competitive athletes and a group of non-dancers/non-athletes ($N = 872$ subjects from 414 families). Consistent with the emotional side of dancing, dancers scored higher on the Tellegen absorption scale (TAS) (Tellegen and Atkinson, 1974; Tellegen, 1982), a questionnaire that correlates positively with spirituality and altered states of consciousness, as well as the reward dependence factor in Cloninger's TPQ (Cloninger, 1987), a measure of need for social contact and openness to communication. Highly significant differences in *AVPR1a* haplotype frequencies (RS1 and RS3), especially when both *SLC6A4* polymorphisms (HTTLPR and VNTR) were also considered in the genetic analysis, were observed between dancers and athletes. Similarly, dancers differed from a group of non-dancers and non-athletes. Thus, dancers differed from both control groups: athletes as well as non-dancers and non-athletes. Association was also observed between TAS scores and *AVPR1a*. Significant association was observed between TPQ reward dependence scores and *AVPR1a*. Therefore, based on the social role of AVP across vertebrates and the association between *AVPR1a* and the TAS and TPQ reward scale in humans, we hypothesized that the association we observed between *AVPR1a* (and *SLC6A4*) and dance reflects the social communication, courtship and spiritual facets of the dancing phenotype, rather than other aspects of this complex phenotype such as sensorimotor integration. Indeed, as discussed below, AVP and OXT play a role in mouse ultrasonic vocalizations (USVs) that are likely to be related to the affiliative actions of these two neuropeptides in facilitating dyadic (mother–pup) and group interactions. Therefore, the role of *AVPR1a* in dance is not surprising as it fits well with the role of this hormone as a facilitator of social and affiliative relationships in other species.

Musical memory

An appreciation for the direct effects of AVP on central nervous system (CNS) function did not emerge until 1965 by the seminal observations of de Wied (1965), who had been investigating the relationships between conditioned behaviour and neuro-endocrine mechanisms. In the often referenced study, de Wied demonstrated that removal of the posterior and intermediate lobes of the pituitary accelerated extinction of conditioned avoidance behaviour in rats without affecting its acquisition, an effect that could be normalized by peripheral administration of crude pituitary extract (pitressin) or lysine vasopressin (de Wied, 1965). This provided the first evidence for a direct effect of vasopressin on CNS function. For inclusive reviews of the role of AVP and OXT as neuromodulators especially of memory processes, see McEwen (2004a, b, c, d).

The role of AVP in memory processes just discussed, and our study of *AVPR1a* and dance (Bachner-Melman et al., 2005a) suggested to us that it would be worthwhile to examine the role of this receptor in musical memory as well. Together with Roni Granot of the Musicology Department at the Hebrew University (Granot et al., 2007), 82 university students were administered an extensive battery of musical and phonological memory tasks. Their scores were examined for an association with promoter repeats in the *AVPR1a* and serotonin transporter genes. Highly significant, gene × gene (epistatic) interactions were observed between promoter-region polymorphisms and musical as well as phonological memory using family-based and population-based tests. Given the prominent role of vasopressin in social behaviour, the preliminary association found in our study between musical memory and vasopressin could serve to support evolutionary accounts postulating a social adaptive role in music (see above) and even early protolanguage.

In infant mice, both neuropeptides, OXT and AVP, contribute to infant USVs. For example, Winslow and Insel showed that central administration of AVP decreased the number of rat pup USV and that co-administration of AVP and receptor antagonists suggested that changes in vocal behaviour were mediated by the V1 receptor subtype (Winslow and Insel, 1993). Conversely, OXT knockout pups emit fewer USVs with maternal separation (Winslow and Insel, 2002). The contrary actions of these two neuropeptides

may not be so unusual. For example, OXT and AVP affect endocrinological systems often with oppositional effects to one another (de Wied et al., 1993). This functional overlap of AVP and OXT has led some researchers to refer to these neuropeptides as 'ying-yang' neurohormones (Legros, 2001). Interestingly, in male mice adults, USVs have the characteristics of song, consisting of several different syllable types whose temporal sequencing includes the utterance of repeated phrases (Holy and Guo, 2005). Individual males produce songs with characteristic syllabic and temporal structure. More recently, it was shown that USV production was positively correlated with the social investigation responses of mice from two genetically differentiated strains. Interestingly, several USV characteristics segregated with the genetic background of young mice, including a higher average frequency and shorter duration for the USVs emitted by B6 mice. However, the possible role of OXT and AVP in adult male vocalizations remains to be investigated.

In the fish, *Porichthys notatus*, vasotocin (evolutionary precursor of vasopressin/oxytocin) produced dose-dependent inhibitions of parameters associated with call initiation (burst latency and number of vocal-motor bursts elicited) but not of vocal-motor patterning (fundamental frequency and burst duration) (Goodson and Bass, 2000). Together, these findings provide support for the suggestion that AVT modulates some of the neuronal processes underlying social/acoustic communication.

It is worthwhile noting that many individuals with autism have been reported to have unusual musical abilities compared to their other abilities. Musical savants, along with their performance skills and prodigious musical memories, possess absolute pitch (AP), and many, if not most, of these savants have autistic features (Miller, 1989). Further, a survey of savant skills in 5400 children with autism suggests that 5% of them have musical savant skills (Rimland and Fein, 1988), so the prevalence of AP among people with autism may be as high as 1 in 20. In a recent study (Heaton et al., 1998), paired single notes with animal pictures was used as an analogy for AP, and it was shown that in comparison to mental-age-matched control subjects, musically naïve autistic children were better able to identify and remember single notes. Brown et al. (2003) showed that musicians with AP show some of the personality, language and cognitive features associated with autism.

From fish to humans, AVP appears to participate in the shaping of a broad 'musical phenotype', including USVs in lower vertebrates and higher functions such as dance and musical memory in humans. Ultimately, vocalization/music appears to be an expression of social communication and affiliative behaviour. Remarkably, the same neuropeptides partially contribute to these varied yet coordinated phenotypes across vertebrates. These conjectures are supported by our provisional findings showing association between dance/musical memory and *AVPR1a* (Bachner-Melman et al., 2005a; Granot et al., 2007), the role of AVP in USVs and affiliative behaviours in fish and mice (Winslow and Insel, 1993; Goodson et al., 2004), the relationship between autistic traits and AP (Brown et al., 2003) and the association between autistic disorder itself and *AVPR1a* (Kim et al., 2002; Wassink et al., 2004; Yirmiya et al., 2006).

Neuroeconomics: *AVPR1a* and altruism/prosocial behaviour

The dictator game

We had previously reported that the dopamine D4 receptor (DRD4) common polymorphisms contributed to individual differences in a self-report questionnaire of altruism or prosocial behaviour (Bachner-Melman et al., 2005b). However, the limitation of self-report questionnaires prompted us to employ a "costly" measure of altruism/prosocial behaviour to substantiate that common polymorphisms indeed contribute to this uniquely human trait. We collaborated with Gary Bornstein (experimental economics) and Ariel Knafo (developmental psychology) and in a seminal paper by Knafo et al. (2008), we examined the contribution of a common genetic polymorphism, the *AVPR1a* RS1 and RS3 repeat regions, to individual

differences in allocation of funds in the so-called dictator game.

Economic games provide a method for observing human behaviour in the laboratory that has many advantages over the standard self-report questionnaires (Camerer and Fehr, 2003). Games recreate social interactions in the laboratory using real money payoffs and thus engage people in 'put your money where your mouth is' decisions. A well-defined game also provides the benefits of quantifiability, replicability and comparability across participants and therefore constitutes a more reliable tool for measuring social decision-making.

A robust body of experimental evidence based on laboratory games shows that human behaviour deviates from economic predictions of profit maximization. A game that best demonstrates this incongruity is the dictator game. The first player, or "dictator", makes a unilateral decision regarding the distribution of a fixed sum of money between herself and the second player, the "recipient". Because the recipient is completely powerless, the dictator is unconstrained by fear of reprisal or other strategic considerations, and her allotment can be seen as a measure of pure altruism (Kahneman et al., 1986; Forsythe et al., 1994).

As such, the dictator game is the most prominent paradigm used by economists to test the existence of altruism (Bolton et al., 1998; Henrich et al., 2001; Camerer and Fehr, 2004). As noted by Eckel and Grossman (1996), the behaviour of subjects in dictator games is well documented and deviates from payoff maximization. Contrary to a strategy of maximizing fitness, participants do donate part of the money, with typical games resulting in around 80% of the participants electing to donate some money and some (about 20%) even splitting the pie evenly (Forsythe et al., 1994). The common explanation given for these observed results is that most participants are motivated by "other regarding preferences" (altruism or fairness) in addition to monetary payoffs.

An attractive candidate that we hypothesized might partially explain individual variance in altruistic giving in the dictator game is the *AVPR1a* gene. We conjectured that allocation of funds in the dictator game may be modulated by trait-influenced patterns of social interactions. Therefore, the *AVPR1a* receptor appeared as a likely candidate for influencing proself vs. prosocial styles of behaviour.

In Fig. 4, we show the allocation of Shekels (₪), out of a 50 ₪ or $12 pie, given to recipients by the 'dictators' in this study. For example, 14.9% of the participants allocated nothing to the 'other', 34.6% allocated half their endowment (25 ₪) and 6.7% allocated the entire sum. Although some subjects allocated intermediary sums, there was a pattern of modal allocating.

As shown in Fig. 5, significantly fewer participants with short versions of the *AVPR1a* RS3 repeat allocated high sums to the 'recipient' than participants with long versions. Additionally, the presence of RS3 long repeats had an additive effect on allocation amounts. Subjects homozygous for short repeats gave 15.4 ₪ whereas subjects homozygous for long repeats gave 22.2 ₪, an effect size of approximately 0.5 S.D.

Participants reported their own prosocial behaviour with the value expressive behaviour scale by Bardi and Schwartz (2003). Two subscales were used, that represent two different aspects of prosocial values. The universalism behaviour subscale taps behaviours that represent a prosocial motivation for understanding, appreciation, tolerance and protection of the welfare of all people (e.g. "make sure everyone I know receives equal treatment"; "donate money for saving people who suffer from war, famine etc. in distant countries"). Significant association was observed between scores on the universalism behaviour subscale and the RS3 repeat.

The other subscale is the benevolent behaviour subscale (Bardi and Schwartz, 2003). This subscale taps behaviours that represent a prosocial motivation to help and support others with whom one is in close or daily social contact (e.g. "agree easily to lend things to neighbours"; "help my friend to perform tasks such as moving and studying"). Significant association was observed between scores on the benevolent behaviour subscale and the RS3 repeat.

We also examined the robustness of our first analysis by using UNPHASED, a procedure that implements a family-based design and avoids the

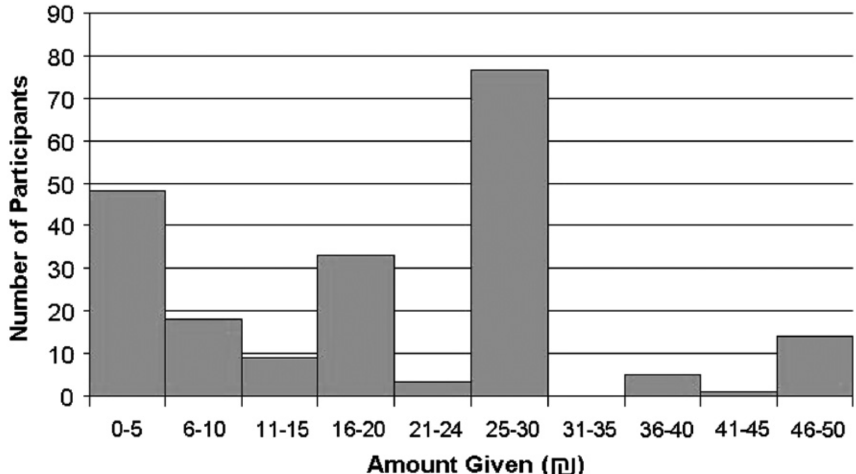

Fig. 4. Distribution of allocation sums by participants in dictator game. 25 ₪ (shekels) was the modal value in this distribution and was used as the cutoff point to divide participants into low and high allocators. Altogether, 46% of the participants were designated as high allocators. The current results can be compared to Forsythe et al. (1994) who explored the replication and statistical properties of the dictator game. The standard perfect equilibrium analysis of the dictator game begins with the assumption that each player prefers more money to less (Bolton and Ockenfels, 2000). In the dictator game, the so-called 'dictator' should keep all the money. However, in the case of the $10 game, 79% did not take the entire sum, with 20% leaving half. The mode of the distribution was $3 or 30%. Notably, Forsythe et al. (1994) showed distributions of dictator giving, which are both anomalous to standard economic theory of maximizing profit as well as robustly replicable.

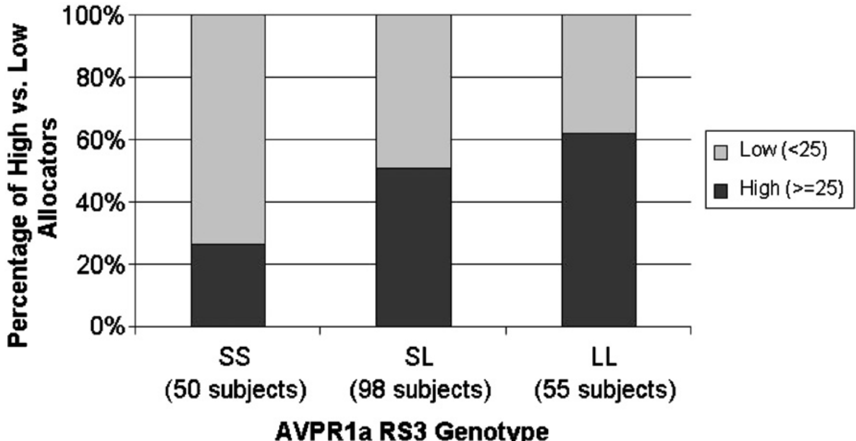

Fig. 5. Mode of giving in the dictator game. High vs. low allocation amounts in ₪ (shekels) grouped by short (308–325 bp) vs. long (327–343 bp) *AVPR1a* RS3 promoter-region repeat length (SPSS v13 Crosstabs two-tailed: likelihood ratio $= 14.75$, $p = 0.0006$, DF $= 2$). Percentages indicate ratio of high (≥ 25 ₪) vs. low (< 25 ₪) allocators for each of the three genotype groupings. We chose the short and long lengths of the RS3 and promoter length so that approximately equal numbers of participants were present in each group. Any other split led to very small groups in either the long or short category for a total sample size of $N = 203$.

conundrum of population admixture or stratification in testing association between money allocations and RS1 and RS3 repeats. As expected from our first analysis, association and dictator game allocations was significant (global $p < 0.05$) only for the RS3 repeat. The third most common allele (12%), 329 bp, showed significant association with allocation ($p = 0.008$).

Importantly, as noted above (Fig. 2), levels of *AVPR1a* mRNA in post-mortem human hippocampal specimens correlates with the length of the RS3 repeat region, suggesting that promoter repeat serves a similar regulatory function in humans as in the vole. Nevertheless, the possibility that RS3 is in LD with other functional polymorphisms in the 5′ upstream region, that might explain some of our results, cannot be excluded.

AVPR1a and infidelity

Cherkas and colleagues investigated the genetics of infidelity in more than 800 twin pairs; after accounting for potential confounds such as divorce, number of sexual partners and age, they reached a heritability estimate of 41% for infidelity. This prompted them to genotype the *AVPR1a*.RS3 on a subgroup of 149 dizygotic twins; however, their results showed no significant

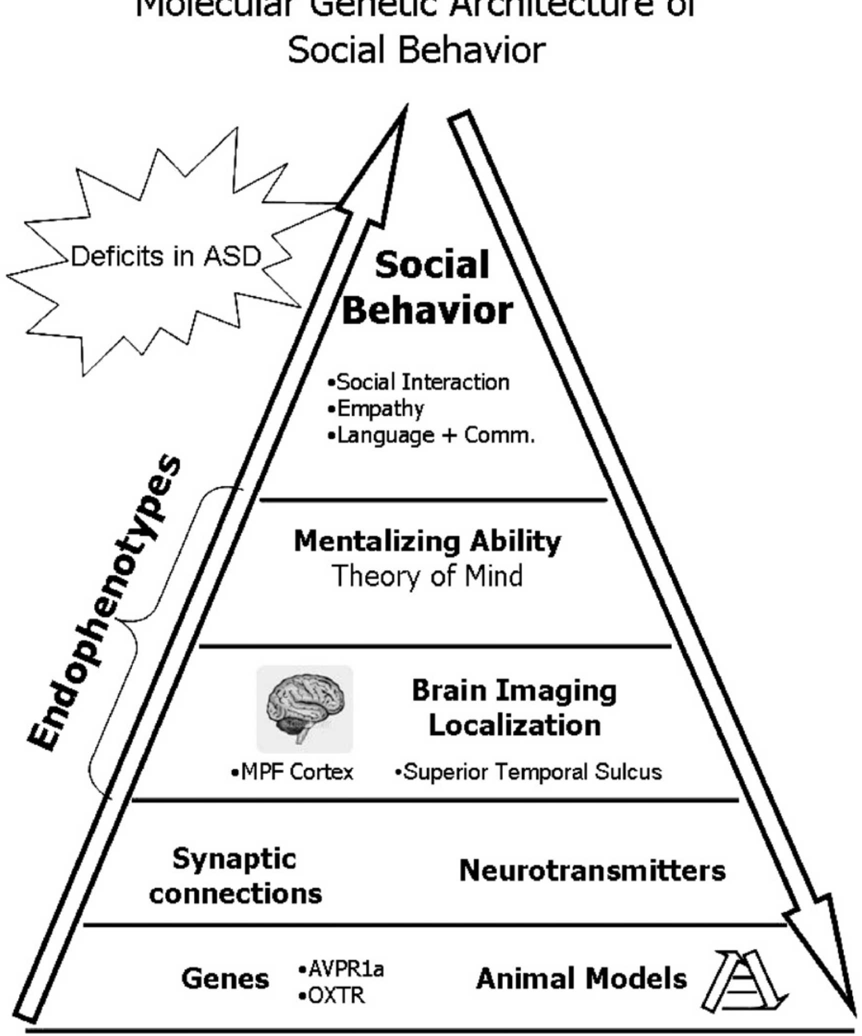

Fig. 6. Molecular genetic architecture of social behaviour.

association between RS3 microsatellite variability and infidelity or number of sexual partners (Cherkas et al., 2004). We suggest that although fidelity may seem to be an indicator of the global endophenotype of prosocial responding, the reason for this null finding may be that infidelity is affected by many additional factors such as sexual arousal.

Future directions

Our research group has for more than a decade sought to understand how common polymorphisms impact on individual differences spanning a broad spectrum of human behaviours (Ebstein, 2006). One of the most intriguing gene families we have studied is the OXT-AVP neuropeptides that across millions of years of vertebrate evolution from fish to man have become important modulators of social communication and affiliative behaviours. Our own studies of these peptides as discussed in this review attest to the diverse phenotypes that polymorphic variants of the *OXTR* and *AVPR1a* genes contribute to. Remarkably, from autism to altruism with musical notes in between, our studies suggest that common polymorphisms in genes coding for elements of AVP and OXT neurotransmission account for some of the individual differences in a super-phenotype of social communication and affiliative behaviour.

Finally, future studies should continue to leverage interdisciplinary strategies to clarify the pathway from genome to higher social behaviour in man. We have summarized our model of the molecular genetic architecture of social behaviour in Fig. 6.

Acknowledgements

This study was supported in part by grants from the Hebrew University (BINCA: GB, RPE, AK, AD), the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities (RPE and GB, NY and RPE), the Israel National Institute for Psychobiology (AK) and Phillip Morris USA and Phillip Morris International (RPE).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 35

Oxytocin and experimental therapeutics in autism spectrum disorders

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Abstract: Autism is a developmental disorder characterized by three core symptom domains: speech and communication abnormalities, social functioning impairments and repetitive behaviours and restricted interests. Oxytocin (OXT) is a nine-amino-acid peptide that is synthesized in the paraventricular and supraoptic nucleus of the hypothalamus and released into the bloodstream by axon terminals in the posterior pituitary where it plays an important role in facilitating uterine contractions during parturition and in milk let-down. In addition, OXT and the structurally similar peptide arginine vasopressin (AVP) are released within the brain where they play a key role in regulating affiliative behaviours, including sexual behaviour, mother–infant and adult–adult pair-bond formation and social memory/recognition. Finally, OXT has been implicated in repetitive behaviours and stress reactivity. Given that OXT is involved in the regulation of repetitive and affiliative behaviours, and that these are key features of autism, it is believed that OXT may play a role in autism and that OXT may be an effective treatment for these two core symptom domains. In this chapter we review evidence to date supporting a relationship between OXT and autism; we then discuss research looking at the functional role of OXT in autism, as well as a pilot study investigating the therapeutic efficacy of OXT in treating core autism symptom domains. Finally, we conclude with a discussion of directions for future research.

Keywords: autism spectrum disorders; oxytocin; vasopressin; experimental therapeutics; treatment; social functioning; social cognition; repetitive behaviours

Introduction

Over the years, a number of researchers have observed that the peptide hormone oxytocin (OXT) may be implicated in autism and related autism spectrum disorders (ASD) given that repetitive behaviours and social deficits are core ASD symptom domains, and that OXT is involved in the regulation of repetitive and affiliative behaviours (Modahl et al., 1992; Panksepp, 1992; Waterhouse et al., 1996; McCarthy and Altemus, 1997; Insel et al., 1999; Hollander et al., 2003; Lim et al., 2005; Bartz and Hollander, 2006; Carter, 2007). In this chapter, we discuss the idea that OXT may be implicated in ASD and review recent efforts to target the repetitive behaviours and social cognition/functioning domains in ASD using intravenous and intranasal OXT administration. Specifically, we begin by providing a brief overview of ASD and its core symptom domains and address why OXT may be relevant to ASD; we then review evidence to date supporting

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DOI: 10.1016/S0079-6123(08)00435-4

a relationship between OXT and ASD, specifically focusing on studies of altered blood plasma levels in ASD, genetic studies involving the OXT and AVP V1a receptors and ASD and our research looking at the functional role of OXT in ASD, as well as a pilot study investigating the therapeutic efficacy of OXT in treating core ASD symptom domains.

Autism spectrum disorders

Autism and ASD are characterized by abnormalities in speech and communication, impaired social functioning and repetitive behaviours and restricted interests; this review will focus on the latter two symptom domains because they are most relevant to OXT. Social interaction impairments are the most characteristic deficits in ASD. These impairments include the failure to use standard non-verbal behaviours to regulate social interactions with others (e.g., gaze aversion when interacting with others, limited range of affective expression, or not directing affective expressions to others and difficulty coordinating gesture with speech to support communication). Failure to share enjoyment, interests and achievements with others is also characteristic, as is a lack of social and/or emotional reciprocity. More generally, individuals with ASD have difficulty engaging in two-way interactions, and interaction partners are often left with the impression that they are aloof, passive or somewhat odd. Finally, awareness of and/or interest in others are often impaired, which can undermine the individual's ability to be empathic. In addition to these general social deficits, individuals with ASD show specific social cognitive deficits, in particular difficulties recognizing faces (Szatmari et al., 1990; Davies et al., 1994; Barton, 2003), and difficulties processing the affective states of others, through both facial displays (Hobson et al., 1988; Tantam et al., 1989) and tone of voice (Hobson et al., 1988; Rutherford et al., 2002).

Repetitive behaviours and narrow, restricted interests define the third symptom domain. These include intense preoccupations that can be abnormal in their intensity, or content. In addition, preoccupation with parts of objects and repetitive behaviour directed at objects are also characteristic, as is rigid adherence to routines and rituals and a desire for sameness. Some individuals also exhibit stereotyped and repetitive motor mannerisms, typically involving the hands (e.g., clapping and finger flipping) or the whole body (e.g., rocking).

Oxytocin and autism spectrum disorders

Oxytocin

As described in detail elsewhere in this volume, OXT is a nine-amino-acid peptide, or nonapeptide. It is synthesized in magnocellular neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus and secreted into systemic circulation (via the posterior pituitary) where it acts as a hormone, facilitating uterine contractions during parturition and milk let-down (Burbach et al., 2006). However, OXT and the structurally similar peptide arginine vasopressin (AVP) are also released within the brain where they act as neuromodulators. In the brain, these neuropeptides are released from multiple sites of the neuronal membrane, but especially dendrites, and can act on relatively distant targets (Landgraf and Neumann, 2004), including the hippocampus, amygdala, striatum, hypothalamus, nucleus accumbens and nuclei in the mid- and hindbrain (Sofroniew, 1983). Importantly, it is through its neuromodulatory role that OXT (and AVP) influences sexual and social behaviour (e.g., mother–infant and adult–adult pair-bonding, social memory/recognition), and other processes including stress–response, emotionality, and learning and memory (Argiolas and Gessa, 1991; Insel, 1992; Engelmann et al., 1996; McCarthy and Altemus, 1997; Insel et al., 1999; Landgraf and Neumann, 2004). As we discuss later, specific OXT receptors that are localized in different brain regions play an important role in the regulation of social behaviour (Breton and Zingg, 1997). OXT receptors are profuse in the limbic system and in the autonomic regions of the brain stem; however, their expression in different brain regions can vary, even in closely

related species, and it is believed that differences in social behaviour can be accounted for, in part, by differences in the distribution of OXT and AVP receptors in the brain.

Oxytocin and repetitive behaviours

Research suggests that OXT is involved in repetitive behaviours. Intracerebroventricular (ICV) administration of OXT has been found to induce such stereotyped behaviours as stretching, repetitive grooming, startle and squeaking in mice (Meisenberg and Simmons, 1983; Drago et al., 1986; Insel and Winslow, 1991; Nelson and Alberts, 1997), grooming in rats (Drago et al., 1986; Van Wimersma Greidanus et al., 1990) and wing-flapping in chicks (Panksepp, 1992). Moreover, OXT has been linked to obsessive-compulsive disorder (OCD) — which is marked by obsessional thinking and/or repetitive behaviours. As McDougle et al. (1999) summarize, in addition to the animal findings just described, reports indicate that pregnancy or the puerperium (periods when OXT levels may fluctuate) is associated with OCD onset or a worsening of symptoms in women. Moreover, adults with non-tic-related OCD were found to have increased cerebrospinal fluid OXT. Finally, as McDougle et al. (1999) note, the OXT system has extensive interactions with the serotonin and dopamine systems, both of which are implicated in OCD.

Oxytocin and social behaviour

As noted, two and a half decades of research suggest that OXT and AVP play a critical role in regulating social behaviour. Most of this research is based on animal studies investigating selective and enduring adult–adult pair bonds and maternal behaviour. In particular, prairie voles are well suited to study the neurobiology of affiliation because they typically form enduring pair-bonds (i.e., are socially monogamous) and display biparental care, and because the closely related montane vole, which tends to be more asocial, allows for comparative investigations (Carter et al., 1995; Insel, 1997; Insel and Young, 2001). OXT — and AVP — have been implicated in the social behaviour displayed by prairie voles. For example, researchers have been able to establish a partner preference in female prairie voles in the absence of mating (mating is typically required for partner preference formation) by centrally administering OXT (Williams et al., 1994; Insel and Hulihan, 1995), and have been able to block the formation of a partner preference in female prairie voles by administering an OXT antagonist prior to mating (Insel and Hulihan, 1995). Interestingly, although differences in the expression of OXT in prairie and montane vole have not been found (Wang et al., 1996); as alluded to earlier, differences in OXT receptor distribution have been reported (Insel and Shapiro, 1992; Insel et al., 1994; Young et al., 1996, 1997). It is thought that these differences in receptor distribution likely underlie differences in social behaviour displayed by these two vole species because these peptides are acting on different brain regions. In prairie voles, OXT receptors are abundant in regions associated with reward processing, reinforcement and conditioning like the prelimbic cortex and nucleus accumbens; by contrast, in montane voles, OXT receptors are less prevalent in these regions, and are more prevalent in other regions like the lateral septum.

Studies of maternal behaviour also support the role of OXT in social affiliation. Although virgin female rats typically find pups to be aversive and actively avoid them (see Fleming and Anderson, 1987), maternal behaviour can be induced by centrally administering OXT (Pedersen et al., 1982), whereas the onset (but not maintenance) of maternal behaviour can be inhibited by centrally administering OXT antagonists (Insel, 1992). In addition, ICV injection of OXT has been found to facilitate maternal behaviour (i.e., acceptance of a foreign lamb) in sheep (Kendrick et al., 1987; Keverne and Kendrick, 1992), one of the few species to display selective maternal care (Insel and Young, 2001). Finally, Olazabal and Young (2006b) observed a positive correlation between maternal behaviour (i.e., time spent crouching over pups) and the density of OXT receptor binding in the nucleus accumbens in juvenile prairie voles, suggesting that OXT influences individual differences in maternal behaviour

within a species; moreover, these researchers found that maternal behaviour could be blocked by administering an OXT antagonist in the nucleus accumbens in adult female prairie voles (Olazabal and Young, 2006a).

Of particular interest to autism and the present chapter is research on the role that OXT and AVP play in social cognition. Rodents primarily rely on olfaction for social recognition; as such, the duration of olfactory investigation is a standard procedure for measuring social recognition in rodents. Using duration of olfactory investigation to measure social recognition, Popik et al. (1992) found that low (but not high) doses of OXT facilitated social recognition in rodents; other researchers have shown that centrally administered OXT antagonists disrupt social memory in female rats (Engelmann et al., 1998). Moreover, studies have found that genetically modified mice that do not produce OXT fail to recognize a novel mouse over repeated exposures (Ferguson et al., 2000; Choleris et al., 2003), but that this deficit can be rescued by a single ICV injection of OXT prior to the initial encounter (Ferguson et al., 2001). Interestingly, social memory deficits have also been observed in mice with a null mutation in the AVP receptor (V1a) gene (*Avpr V1a*) (Bielsky et al., 2004); as with the OXT knockout mice, this social memory deficit can be rescued through site-specific treatment with a vasopressin receptor (AVPR) (V1a) viral vector in the lateral septum, which renews AVPR (V1a) binding in the targeted area (Bielsky et al., 2005).

Although research examining the effects of OXT on human social behaviour has been limited — mainly because of the methodological difficulties involved — a few recent studies suggest that OXT may be involved in aspects of trust, social cognition and regulating responses to social stimuli. Kosfeld et al. (2005) administered intranasal OXT or placebo to male volunteers who then played the "trust game" in which they were given a sum of money and were given the opportunity to invest portions of that money with an anonymous partner; investing in the other player could lead to higher payoffs for both players, but the investor always ran the risk that the trustee may violate that trust. Intranasal OXT significantly increased trust among participants compared to placebo. Another study used functional magnetic resonance imaging (fMRI) to investigate changes in amygdala activity in male participants who received intranasal OXT or placebo and then viewed fear-inducing stimuli of a social (angry and fearful faces) and non-social (threatening scenes) nature; reduced amygdala activity in response to the fearful stimuli was observed following OXT compared to placebo (Kirsch et al., 2005). Finally, Domes et al. (2007) found that OXT facilitated performance on the Reading the Mind in the Eyes test, which assess the ability to infer the mental states of others — this finding is of particular relevance to ASD because individuals with ASD often show deficits in Theory of Mind, and on this task in particular (Baron-Cohen et al., 2001).

The oxytocin–autism link

In light of the findings described above implicating OXT in repetitive behaviours and aspects of social functioning, researchers have speculated that OXT may be implicated in autism given that these are two core symptom domains of the disorder (Modahl et al., 1992; Panksepp, 1992; Waterhouse et al., 1996; McCarthy and Altemus, 1997; Insel et al., 1999; Hollander et al., 2003, 2007; Lim et al., 2005; Bartz and Hollander, 2006; Lim and Young, 2006; Carter, 2007). In support of this notion, lower blood plasma OXT levels have been observed in children with autism compared to age-matched controls (Modahl et al., 1998); moreover, the plasma samples obtained from the autistic children were associated with higher OXT precursor levels, as well as an increased ratio of OXT precursor to OXT, suggesting that OXT may be processed differently in the brains of children with autism (Green et al., 2001). Another study, however, found higher OXT plasma levels in adults with ASD compared to controls (Jansen et al., 2006); the reason for this discrepancy is unclear, however, as the authors suggest, developmental factors may contribute to the observed increased basal OXT levels in adulthood.

Genetic studies also support a role of OXT in ASD. Ylisaukko-Oja et al. (2006) conducted a

combined analysis of primary genome scanned data to identify potential susceptibility loci for autism; findings from this study implicate the region of chromosome 3 — in which the OXT receptor gene (*Oxtr*) is located — as a susceptibility loci for ASD. Wu et al. (2005) found an association between two single nucleotide polymorphisms in the *Oxtr* and ASD in a sample of Han Chinese family trios. Finally, Jacob et al. (2007) also found an association between the *Oxtr* and autism in a small Caucasian sample; specifically, a significant association was detected at rs2254298 but, in contrast to Wu et al., not at rs53576. Interestingly, polymorphisms in the AVP receptor gene (*Avpr*) have also been linked to autism. Kim et al. (2002) found evidence for disequilibrium between autism and one microsatellite polymorphism of the *Avpr (V1a)* gene; Wassink et al. (2004) also found evidence for linkage disequilibrium in the *Avpr (V1a)*; and Yirmiya et al. (2006) demonstrated a link between the *Avpr (V1a)* gene and autism.

Targeting the repetitive behaviours and social cognition symptom domains

Drawing upon the large animal literature implicating OXT in repetitive behaviours and affiliation, as well as research by Modahl et al. (1998), Hollander and colleagues have been interested in investigating the functional role of OXT in ASD, as well as the potential therapeutic value of OXT in treating core ASD symptom domains.

Intravenous OXT challenge and repetitive behaviours

To investigate the role of OXT with respect to repetitive behaviours in ASD, Hollander and colleagues conducted a double-blind, placebo controlled, crossover study in which synthetic OXT (Pitocin) was administered to 15 adults with ASD via intravenous infusion in a randomized, counter-balanced fashion (Hollander et al., 2003). Each subject served as his or her own control and completed both OXT and placebo challenges on separate days; synthetic OXT or placebo was continuously infused over a 4-h period following an overnight fast (see Hollander et al., 2003, for a complete description of the methodology).

Frequency of repetitive behaviours were assessed using a four-point ordinal scale ranging from 0 (*never*) to 3 (*constantly*) at baseline (0), 60, 120, 180 and 240 min over the course of the laboratory challenge. A repeated measures analysis of variance revealed that the frequency of repetitive behaviours was reduced over time following OXT administration compared to placebo. In addition, a decrease in the total number of different repetitive behaviours was observed following OXT administration compared to placebo. Reported side effects from OXT infusion were mild and included drowsiness, anxiety, depression, headache, tingling, backache, trembling, restlessness, stomach cramps and enuresis.

This study suggests that OXT may have value in treating repetitive behaviours in ASD; however, it also raises some interesting questions. As noted, OXT administration in animals actually increases stereotyped behaviours. Moreover, two early studies investigating the therapeutic efficacy of OXT in treating obsessive-compulsive symptomatology in patients with OCD found no support for the therapeutic efficacy of OXT (den Boer and Westenberg, 1992; Epperson et al., 1996). Given these observations, why would OXT reduce repetitive behaviours in ASD? We believe it is important to keep in mind that the animal studies examined the relationship between OXT and repetitive behaviours in a normal, intact animal model, not in an altered system as is believed to be the case in autism. With respect to the negative findings regarding OXT treatment of OCD, it may be that too much OXT or an increased sensitivity to OXT is implicated in OCD, whereas a deficit of OXT is implicated in ASD (as suggested by Modahl et al.'s research). Moreover, recent research suggests that repetitive behaviours in autism may differ from those in OCD, not only phenomenologically, but also in terms of underlying neurobiology and, possibly, genetics (Anagnostou et al., 2005). Thus, OXT may have different effects in the treatment of repetitive behaviours in autism compared to repetitive behaviours characteristic of OCD.

Intravenous OXT challenge and social cognition

We also have preliminary data demonstrating the beneficial effects of intravenous OXT on the aspects of social cognition in ASD; in particular, ability to assign affective significance to speech (Hollander et al., 2007), a deficit that is present in many individuals with ASD and that is thought to be central to the social and speech deficits in ASD (Gervais et al., 2004; Kuhl et al., 2005). Specifically, comprehension of affective speech (Heilman et al., 1975) was tested at baseline (just prior to the OXT infusion), and at 30, 60, 120, 180 and 240 min over the course of the infusion (see above study for methods description). In this task, participants were presented with four pre-recorded sentences of neutral content (e.g., "The boy went to the store"); each sentence was presented with one of four emotional intonations (*happy*, *indifferent*, *angry* and *sad*), with the pairing of emotional expression and sentences in 1 of 6 counterbalanced orders. Participants indicated the emotional mood of the speaker by pointing to the word that corresponded to the emotion that they believed matched the one they heard on the tape.

Data were first transformed and scored dichotomously as 1 (*all items correct*) and 0 (*not all items correct*) to account for negative skew and to better balance task difficulty. Mixed regression analysis was used to estimate linear trends in speech comprehension performance for each subject; these individual linear trends were used as the dependent variables. As depicted in Fig. 1, subjects who received OXT first showed increased levels of retention on the task, and did not show a tendency to revert to baseline when retested after a delay of approximately 2 weeks, whereas subjects who received placebo first tended to revert to baseline after the delay. Although this study did not observe a direct effect of OXT on the comprehension of affective speech task (possibly because ceiling effects obscured our ability to detect such effects), these findings are nonetheless consistent with the aforementioned study by Domes et al. (2007) in which intranasal OXT facilitated participants' ability to identify emotion through facial stimuli.

Pilot treatment study of intranasal OXT in autism spectrum disorders

In sum, these data support the potential role of OXT in ASD and suggest that OXT may have therapeutic benefits for the treatment of repetitive behaviours and social deficits; however, research is needed to determine the feasibility and long-term therapeutic effects of OXT for the treatment of ASD. To this end, our group has a pilot study underway investigating the effects of intranasal OXT on core ASD symptoms (i.e. repetitive behaviours, social functioning and social cognition) and global functioning in adults with ASD.

This pilot study is on-going, but preliminary data are promising. Eight high functioning patients meeting criteria for an ASD based on DSM-IV diagnoses and the Autism Diagnostic Interview were randomized to intranasal OXT (IN-OXT) or placebo conditions. Beginning and average end-point dosing was 24 IU bid (i.e. three puffs/nostril twice daily, morning and noon). Reported side effects were minimal per treating clinician adverse events record forms. One patient, randomized to IN-OXT, reported side effects which included increased fatigue and sneezing, which were mild in nature. Analyses indicate that IN-OXT treatment vs. placebo resulted in improvements in repetitive behaviours in this small preliminary data set (E. Anagnostou, personal communication, May 2, 2007). In addition, analysis of responders and non-responders based on the Clinician's Global Impressions-Improvement (CGI-I) scale ratings suggest that more IN-OXT subjects were categorized as responders than placebo subjects; similarly, responders analysis of CGI-I, (Social) ratings suggest that more IN-OXT subjects were categorized as responders than placebo subjects (Bartz et al., 2006; Anagnostou et al., 2007; Bartz et al., 2007).

In addition to these primary outcome measures, preliminary analyses revealed intriguing findings on the Diagnostic Analysis of Non-Verbal Accuracy (DANVA2), which measures emotion recognition across multiple modalities (facial expression, paralanguage or tone of voice and posture). Specifically, improvements from baseline for the IN-OXT group are emerging on some DANVA2

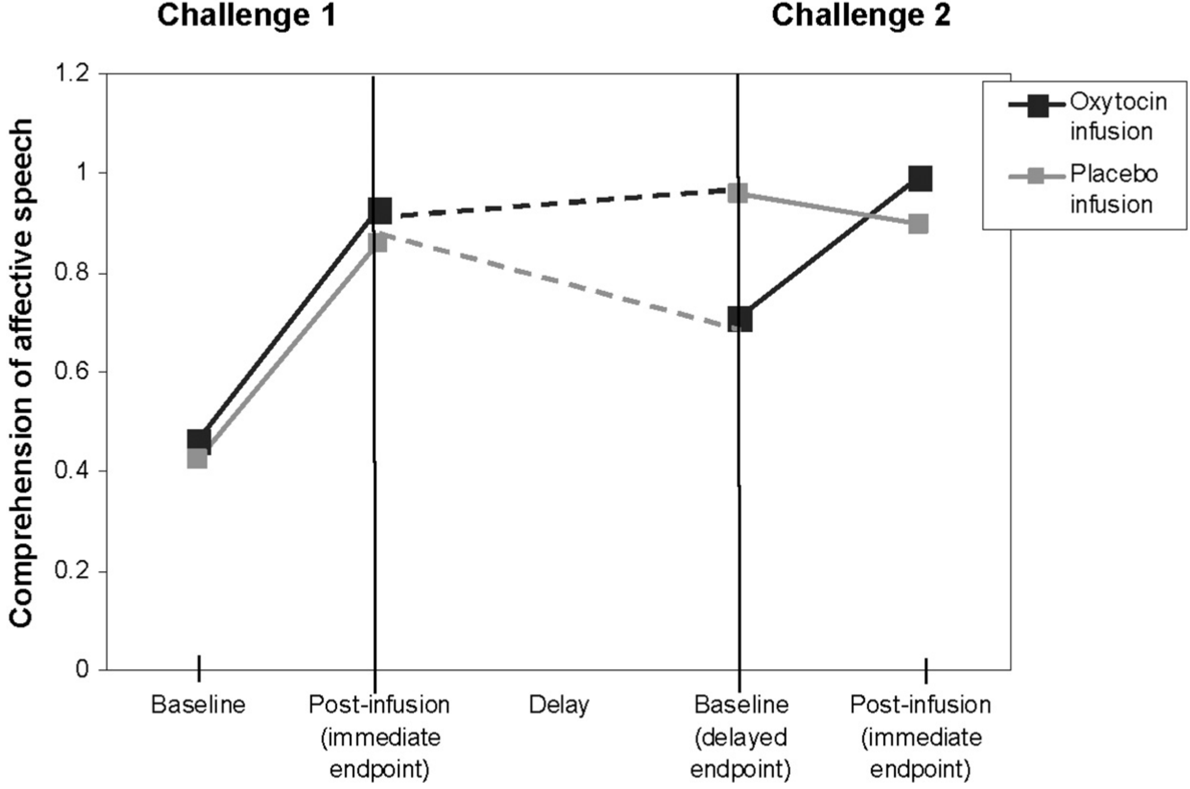

Fig. 1. Mixed regression analysis of predicted linear trends across time of affective speech comprehension as a function of condition (oxytocin vs. placebo) and order of administration (oxytocin 1st vs. placebo 1st). Adapted with permission from Elsevier; Hollander et al., 2007.

subscales (those using child stimuli which may be more challenging for the adults in this study to interpret) (Anagnostou et al., 2007; Bartz et al., 2007) and (E. Anagnostou, personal communication, May 2, 2007). These findings — if they continue to hold — replicate and extend our findings from the challenge study (Hollander et al., 2007) in that they show that IN-OXT has a direct effect on social information processing; these findings are also consistent with the aforementioned findings observed by Domes et al. (2007).

Finally, Event Contingent Records (ECR), a diary methodology that allows participants to report on symptoms, affect, thoughts and behaviour close in time to experience, was included as an exploratory measure to track changes in participants' social functioning in the real world. A subset of participants in this pilot study completed ECR forms for a 4-day period at baseline and a similar period at week 6. Preliminary analyses indicate that fewer cold/aloof behaviours (e.g., "I ignored the other's comments") were indorsed in social interactions in the IN-OXT compared to placebo group (Bartz et al., 2007), suggesting that OXT has the potential to influence participants' behaviour and affective experience in their real life social interactions.

Functional magnetic resonance imaging study of the effects of intravenous OXT in adults with ASD

In addition to looking at the behavioural effects of OXT in ASD, it will also be important to investigate the neural correlates of OXT in ASD. Animal studies indicate that differences in social behaviour appear to be due, at least in part, to differences in the distribution of OXT and AVP receptors in the brain, both within and across

species. To date, no one has yet been successful in developing ligands for OXT and AVP that could be used to identify the distribution of OXT and AVP receptors in the human brain. Although unable to detect specific receptor location, fMRI can be used to identify the neural correlates of OXT in ASD, which can shed light on how OXT is exerting its effects on behaviour, and can be used as a biomarker of treatment response. Hollander and Anagnostou are currently using fMRI to study the effects of intravenous OXT on response inhibition (a proxy for repetitive behaviours) and face processing in adults with ASD. Very preliminary data suggest greater prefrontal cortex and fusiform face area activation post-OXT infusion compared to pre-infusion for the subject that received OXT.

One question arising from this research concerns the issue of exogenous administration and brain penetration. As noted, the effect of OXT on social behaviour is due to its role as a neuromodulator in the brain. Moreover, endogenous OXT in the plasma cannot cross the blood brain barrier (BBB) (Landgraf and Neumann, 2004). So how does OXT, administered via intravenous infusion, exert its effects on repetitive behaviours and social cognition as Hollander et al. (2003, 2007) observed? Most likely, a small amount of exogenous OXT crosses the BBB following intravenous infusion. This is consistent with the notion proposed by Landgraf and Neumann (2004) that transport across the BBB of exogenously administered OXT can result in functionally significant effects if plasma concentrations of exogenous OXT exceed a certain threshold. Indeed, supporting this idea, Jin et al. (2007) showed that peripherally administered OXT could rescue maternal nurturing and social memory deficits in *CD38* mutant mice. Similarly, Ring et al. (2006) showed that peripherally administered OXT could produce anxiolytic effects in male mice that were comparable to the effects achieved by central administration (although substantially larger doses were required for peripheral administration to achieve such effects). Moreover, Ring et al. (2006) showed that a centrally administered OXT antagonist (that does not cross the BBB) can fully reverse the anxiolytic effects of peripherally administered OXT, providing further support for the claim that peripherally administered OXT can penetrate the brain. Intranasally administered OXT, by comparison, is likely directly transported from nasal mucosa to the cerebrospinal fluid, which acts as a mechanism to transport exogenous neuropeptides across the ependyma into the brain parenchyma (Bittencourt and Sawchenko, 2000).

Future directions

In conclusion, tremendous progress has been made over the years in identifying the neurobiology of attachment and social behaviour, and this research has implications for understanding and treating disorders marked by deficits in social functioning like ASD. In particular, OXT and the structurally similar peptide hormone AVP have been implicated in social motivation, pair-bond formation and other aspects of attachment. Drawing upon this literature, our program of research embraces a translational approach to understand core ASD symptom domains and to identify novel treatments for core ASD symptoms. We have found that OXT plays a role in repetitive behaviours and aspects of social cognition in ASD, and preliminary findings suggest that OXT may have value in treating these core ASD symptom domains. Research investigating the effects of OXT in humans, however, is in its infancy and more research is needed in this area. Below we outline some unresolved questions and directions for future research.

Research by Hollander et al. (2007) and by Domes et al. (2007) suggests that OXT facilitates social information processing through two different sensory modalities — that is, through auditory and visual modalities, respectively. This raises questions about mechanism of action. In particular, *how* does OXT facilitate social cognition? Previous research indicates that OXT plays a role in regulating stress and fear reactivity. Thus OXT may facilitate the ability to process social information by reducing anxiety that is inherent in many social encounters. This explanation, in fact, is particularly relevant to ASD because individuals with ASD often report experiencing anxiety when processing social information. Alternately, OXT may increase peoples' motivation to attend to

social cues in their environment by reinforcing social information processing. This hypothesis is supported by research showing that differences in social behaviour in prairie and montane voles can be explained in part by where OXT and AVP are acting in the brain. Future research will most certainly want to investigate this question in humans as it may shed light on the pathophysiology of ASD, as well as possible targets for intervention.

In addition, although genetic studies point to alterations in the *Avpr (V1a)* and risk for ASD, no one has looked at the functional role of AVP in ASD, or the potential therapeutic value of AVP in treating core ASD symptoms. Given the functional overlap between OXT and AVP, and that they are able to influence each other's receptors and functions (Engelmann et al., 1996; Cho et al., 1999; Bales et al., 2004; Landgraf and Neumann, 2004; Ragnauth et al., 2004; Pedersen and Boccia, 2006), it will be important for future researchers to systematically assess the role of both OXT and AVP in ASD. Finally, more research is needed to investigate the OXT and AVP systems in humans across development, especially in the context of ASD. Given that ASD is a developmental disorder, with onset occurring prior to three years of age, it is likely that abnormalities in these systems arise relatively early in development. Along these lines, Carter (2007) proposed the intriguing theory that the male vulnerability to ASD (ratio of males to females with ASD is approximately 4:1) may be due to disruptions in the AVP system, and possibly an excess of AVP, during development because AVP is androgen dependent and AVP plays a key role in male behaviour. If disruptions occur in the AVP system during development (possibly due to increased exposure to androgens), males may be more sensitive to these disruptions, leading to developmental traits associated with ASD (i.e., reduced social behaviour, repetitive behaviours and anxiety). By contrast, Carter (2007) argues, females may be protected from ASD because of their independence and/or insensitivity to the AVP system (many of the processes controlled by AVP in males rely on OXT in females), and/or because of potential protective factors associated with OXT and/or oestrogen.

Finally, it may be useful to consider other factors that influence the functioning of the OXT and/or AVP systems. Recently, Jin et al. (2007) showed that *CD38*, a transmembrane receptor with ADP-ribosyl cyclase activity, plays a key role in regulating complex social behaviour (social recognition and maternal nurturing) by regulating OXT secretion. Thus, variations in *CD38* activity may be associated with individual differences in social cognition and social functioning that characterize individuals with ASD (Bartz and McInnes, 2007; Young, 2007).

Abbreviations

ASD	autism spectrum disorders
AVP	arginine vasopressin
Avpr (V1a)	vasopressin receptor 1a gene
AVPR	arginine vasopressin receptor
CD38	CD38 gene
CGI-I	clinician's global impressions-improvement scale
DANVA2	diagnostic analysis of non-verbal accuracy
ECR	event contingent records
fMRI	functional magnetic resonance imaging
ICV	intracerebroventricular
IN-OXT	intranasal OXT
OCD	obsessive-compulsive disorder
OXT	oxytocin
Oxtr	oxytocin receptor gene
PVN	paraventricular nucleus
SON	supraoptic nucleus

Acknowledgements

The authors gratefully acknowledge the Beatrice and Samuel A. Seaver Foundation and the NIH 5 U54 MH066673-03 STAART Autism Centers of Excellence for their support of the research described in this review. Eric Hollander is listed as an inventor on a patent for Treatment of Autism and Similar Disorders with Oxytocin.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 36

Impact of prosocial neuropeptides on human brain function

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Abstract: Oxytocin and vasopressin are key effectors of social behaviour (Insel, T. R. and Fernald, R. D. (2004). Annu. Rev. Neurosci., 27: 697–722). Oxytocin effects in humans were recently demonstrated by a behavioural study showing selectively increased trust after hormone administration (Kosfeld, M., et al. (2005). Nature, 435: 673–676). Since this suggested involvement of the amygdala, which is linked to trust (Winston, J. S., et al. (2002). Nat. Neurosci., 5: 277–283) — presumably because of its role in danger monitoring — and highly expresses oxytocin receptors (Huber, D., et al. (2005). Science, 308: 245–248), we studied amygdala circuitry after double-blind crossover intranasal application of placebo or oxytocin (Kirsch, P., et al. (2005). J. Neurosci., 25: 11489–11493). Oxytocin potently reduced amygdala activation and decreased coupling to brainstem regions implicated in autonomic and behavioural manifestations of fear, indicating a neural mechanism for the effects of oxytocin in social cognition in humans and providing a potential therapeutic approach to social anxiety currently being tested in social phobia and autism. Furthermore, these data suggested a translational genetic approach. Preliminary findings (data not presented) from our laboratory using imaging genetics indeed implicate genetic variants for both *AVPR1A*, encoding the primary receptor of vasopressin in brain, and the oxytocin receptor, *OXTR*, in amygdala regulation and activation. Taken together, our results indicate neural mechanisms for human social behaviour mediating genetic risk for autism through an impact on amygdala signalling and provide a rationale for exploring therapeutic strategies aimed at abnormal amygdala function in this disorder and in social dysfunction in general.

Keywords: amygdala; brain stem; fMRI; anxiety; imaging genetics

Well-being and survival in primates, including humans, depends critically on social interactions (Silk et al., 2003), and disturbed social behaviour is a key component of diseases such as autism, schizophrenia and anxiety disorders (Adolphs, 2003). Despite the obvious relevance of this domain to medicine and, potentially, innovative therapies, little work has yet been done delineating molecular mediators of social behaviour in human brain. This is in marked contrast to the tremendous interest and work in social cognitive neuroscience, a new research field that investigates cognitive sub-processes for social information processing and their neural correlates (Adolphs, 2003; Insel and Fernald, 2004). It is therefore an interesting research question whether this "top-down", cognitive approach to social behaviour can

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DOI: 10.1016/S0079-6123(08)00436-6

be complemented by ''bottom-up'' approaches that investigate molecular mechanisms and their neural instantiation as a way to uncover specific neurobiological factors shaping the human social brain.

The prosocial neuropeptides, vasopressin and oxytocin, are evolutionarily highly conserved key modulators of complex emotional and social behaviours such as attachment (Insel and Young, 2001), social exploration, recognition (Winslow and Insel, 2004) and aggression (Bosch et al., 2005), as well as anxiety (Liebsch et al., 1996; McCarthy et al., 1996; Appenrodt et al., 1998), fear conditioning (Stoehr et al., 1992) and extinction (Ibragimov, 1990). Dissecting the neural mechanism through which they act therefore represents, potentially, a privileged window into brain function related to social behaviour from a molecular, ''bottom up'' angle. Second, since many aspects of social function are highly heritable (Scourfield et al., 1999), a genetic approach (detailed below) focused on neuropeptides is also a promising approach to identify molecular- and systems-level mechanisms of social cognition in humans.

Although the circuitry of social behaviour is likely to be complex, a convenient starting point and likely focus of this work is the amygdala (Adolphs, 2003), a key mediator of neural functions related to anxiety (LeDoux, 2000) and fear extinction. The lateral nucleus of the amygdala receives and integrates sensory and prefrontal/limbic inputs and then excites, possibly indirectly, neurons in the central nucleus that evoke fear responses via their projections to brain stem regions including periaqueductal grey and reticular formation (LeDoux, 2000). In humans, fearful faces potently activate amygdala (Whalen et al., 1998), lesions of the amygdala impair recognition of fearful faces and lead to social disinhibition (Adolphs et al., 2005), and decreased amygdala activation has been linked to genetic hypersociability (Meyer-Lindenberg et al., 2005) and increased aggression (Kiehl et al., 2001), while increased activation is observed in social avoidance and phobia (Stein et al., 2007). Consistent with the assumption that amygdala activation represents a danger signal in social interaction (Amaral, 2003), both conscious and implicit distrust of faces predicts amygdala activation (Winston et al., 2002) and trust is increased when amygdala is damaged (Adolphs et al., 1998). In humans, evidence for oxytocin binding to amygdala is lacking, while vasopressin binds to amygdala (Loup et al., 1991). However, animal models strongly suggest that the central role of oxytocin in mediating complex social behaviour depends on the function of the amygdala: oxytocin acts on the amygdala to reduce fear (McCarthy et al., 1996) and modulate aggression (Bosch et al., 2005), and mouse knockouts for the oxytocin receptor in mice show a profound social recognition deficit despite normal olfactory and spatial learning abilities that can be fully restored by injection of oxytocin in the medial amygdala (Ferguson et al., 2001). Based on these data, we hypothesized that oxytocin would reduce amygdala activation in humans and modulate its participation in functional networks related to fear processing.

As specified elsewhere in this volume, oxytocin effects in humans were recently demonstrated by a behavioural study showing selectively increased trust after hormone administration (Kosfeld et al., 2005). Since this suggested once again involvement of the amygdala, which is linked to trust (Winston et al., 2002) — presumably because of its role in danger monitoring — and highly expresses oxytocin receptors at least in certain species (Huber et al., 2005), we studied amygdala circuitry after double-blind crossover intranasal application of placebo or oxytocin (Kirsch et al., 2005).

We employed two visually matching tasks requiring perceptual processing of threatening visual stimuli of different social valence that reliably engage amygdala and have been shown to be sensitive to genetic mechanisms of abnormal social behaviour (Meyer-Lindenberg et al., 2005a) and risk for anxiety (Pezawas et al., 2005). In the first task, one of two simultaneously presented angry or afraid faces was matched with an identical target face (Hariri et al., 2002a). In the other task, participants matched one of two simultaneously presented fearful/threatening scenes with an identical target scene (Hariri et al., 2003). As a control, participants matched simple shapes (circles or ellipses). The scenes were selected from a larger set to be devoid of social interaction or

facial displays and should therefore represent non-socially relevant threatening stimuli, while threatening and angry faces are socially relevant stimuli, an assumption supported by previous research with this paradigm (Meyer-Lindenberg et al., 2005a). In a double-blind experiment, 15 participants (age 26.7 ± 3.0, 13 right handers) applied oxytocin or placebo intranasally, a modality shown to reliably deliver neuropeptides to the brain (Knafo et al., 2007). In behavioural testing, the neuropeptide had no effect on task performance, anxiety scales or arousal, in agreement with previous reports (Kosfeld et al., 2005). A nominally significantly worse mood before drug application in the oxytocin condition was observed in one scale but was not confirmed in a second mood scale applied simultaneously. In the fMRI experiment, strong, right-lateralized activation of the amygdala to both classes of stimuli was observed during the placebo condition (Fig. 1), confirming previous results (Hariri et al., 2002a). Compared to placebo, oxytocin significantly depressed amygdala activation (Fig. 1; maximum effect at right amygdala, coordinates 24, 3, −24, $T = 2.59$, $p < 0.006$). Testing the stimulus types separately showed that this effect was more pronounced for faces (socially relevant stimuli) than scenes (Fig. 1). However, this did not represent an interaction as the slope of the effect was almost identical for the two stimulus classes (Fig. 1).

Our finding of reduced amygdala activation under oxytocin confirmed our hypothesis about a contribution of reduced danger signalling to increased trust. In addition, reduced amygdala

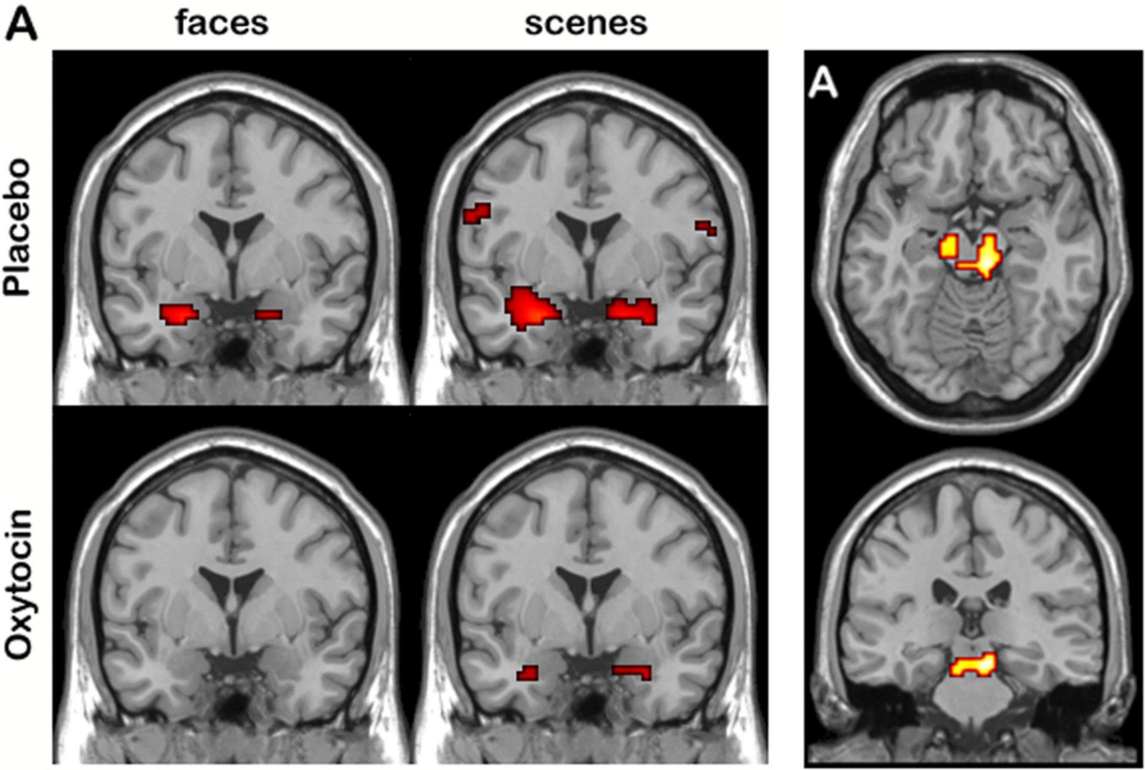

Fig. 1. Left: Oxytocin effects on amygdala activation ($p < 0.01$, uncorrected), rendered on normal coronal MRI at the level of the anterior commissure (in radiological orientation: brain left is on viewer's right). Response to face stimuli left, scene stimuli right. Top, placebo; bottom, oxytocin. Right: Significant decrease in coupling of amygdala to midbrain under oxytocin. Maps of significant ($p < 0.01$, uncorrected) difference in connectivity from amygdala to midbrain, in radiological orientation. Results and component figures from Kirsch et al., 2005. (See Color Plate 36.1 in color plate section.)

activation to fearful faces has been linked to increased sociability and decreased social fear in humans (Adolphs et al., 2005; Meyer-Lindenberg et al., 2005a), and the neuropeptide action observed here may therefore contribute to the prosocial effects of oxytocin. It is intriguing to note that the reduction of amygdala activation was more pronounced for socially relevant stimuli (faces) than for the socially less relevant scenes; while this effect did not reach statistical significance and did not represent an interaction effect, differential impairment of amygdala signalling related to the social relevance of the stimuli is in agreement with emerging primate lesion (Prather et al., 2001) and human (Meyer-Lindenberg et al., 2005a) data indicating that social and non-social fear may depend on dissociable neural systems. Since most human data suggest a relationship of impaired amygdala structure and function with aggressive behaviour (Kiehl et al., 2001), especially in males, our data would seem to be in agreement with observations of oxytocin-induced increased aggression (Bosch et al., 2005). However, expression of aggression is also modulated by situational specifics such as gender (Insel and Fernald, 2004) and status (Winslow and Insel, 1991): reduced amygdala danger signalling might enhance aggression if perception of a fearful victim fails to inhibit aggressive behaviour, but might also reduce reactive aggression triggered by a fear-inducing conspecific.

Given the link between amygdala signalling and fear (Amaral, 2003; Adolphs et al., 2005), our data fit well with the reported anxiolytic effects of oxytocin (McCarthy et al., 1996; Domes et al., 2007b) and its mediation by the amygdala (McCarthy et al., 1996; Bosch et al., 2005) and suggest a potential treatment approach towards socially relevant fear. In addition to the effects on amygdala activation, an important additional mechanism that could underlie the effect of oxytocin in fear processing was recently demonstrated when distinct neuronal populations in the central amygdala activated by neuropeptides were identified, through which oxytocin (but not vasopressin) disrupted the common output from amygdala to the brainstem effector sites of the autonomic nervous system (Huber et al., 2005). While the resolution of fMRI is not currently sufficient to explore intra-amygdalar processing, we investigated whether functional coupling of the amygdala to the brainstem was influenced by oxytocin. To this end, we studied the functional connectivity of the amygdala, a measure of correlated activity in blood oxygen level-dependent (BOLD) over time that is widely used as a simple and robust characterization of functionally relevant neural interaction (Friston et al., 1993).

We found that amygdala was functionally connected to the upper brainstem during the placebo condition and that this connectivity was significantly reduced under oxytocin, in excellent agreement with the data acquired in animal models (Fig. 1, right). Our results indicate that the effect of oxytocin on anxiety may be due to an effect on both amygdala activation and coupling to regions mediating fear response. In agreement with our findings, autonomic response to aversive pictures has been reported to be reduced under oxytocin (Pitman et al., 1993), compatible with the effect on amygdala connectivity seen here.

Summarizing these results, oxytocin potently reduced amygdala activation and decreased coupling to brainstem regions implicated in autonomic and behavioural manifestations of fear, indicating a neural mechanism for the effects of oxytocin in social cognition in human brain and providing a potential therapeutic approach to social anxiety currently being tested in social phobia and autism. Our findings have recently been replicated, both with regard to activation and connectivity (Domes et al., 2007b). In this study, it was also shown that not only fearful but also angry and happy facial expressions were reduced. Together with our results on the differential impact of stimulation with scenes and faces, this supports a concept that amygdala is broadly involved in emotional signalling related to social function, and not specifically to fearful emotion. Interestingly, the same group has also recently shown that oxytocin improves performance on a "mind reading" task in which emotional states of others have to be inferred based on pictures of the eyes (Domes et al., 2007a). Since dampening of the amygdala, if anything, would predict reduced performance in this task as the automatic emotional salience mediated by this structure would be

expected to be reduced, this suggests that cortical areas are also impacted by neuropeptide signalling in humans, an interesting domain for further studies.

Nevertheless, the results discussed clearly implicated amygdala circuitry in neuropeptide function in human brain. Based on these findings, we therefore decided to next examine neural effects of genetic variants, previously associated with autism (Kim et al., 2002; Ylisaukko-oja et al., 2006), in the receptors for oxytocin and its sister neuropeptide vasopressin, to further characterize prosocial neural mechanisms and continue our genetic dissection of the human social brain.

Autism is an especially interesting and promising point of departure as it is a very highly heritable disorder in which, from the initial description of the disorder by Kanner to the currently used diagnostic definition, impairments in social interaction have been regarded as a central feature, suggesting a potential role of prosocial neuropeptides in the pathogenesis and treatment of this common and devastating psychiatric disorder (Insel, 1997; Hammock and Young, 2006). Given the high heritability of autism, this implies that genetic variation related to the neuropeptide system could contribute to the disorder. Several lines of evidence suggest that this is indeed the case. First, genetic studies implicate the human *AVPR1A* gene, encoding AVPR1A, which is located on chromosome 12q and contains two exons that are separated by a 2.2 kb intron, in autism. The 5′-flanking region of the gene contains three polymorphic microsatellite repeats (Thibonnier et al., 2000). Of these, RS3, a complex repeat of $(CT)_4$-TT-$(CT)_8$-$(GT)_n$ 3625 base pairs (bp) upstream of the transcription start site, with 16 different alleles in the population, and RS1, a $(GATA)_n$ repeat with 9 alleles located 553 bp from the start site, have shown nominally significant transmission disequilibrium in autism (Kim et al., 2002; Wassink et al., 2004; Yirmiya et al., 2006). Specifically, Kim et al. (2002) found overtransmission of the 334 and 340 bp alleles of RS3 to probands with autism, and Wassink et al. (2004) found undertransmission of the 312 bp allele of RS1, while the 320 bp allele was overtransmitted in a sub-group of autistic probands with preserved language. Finally, Yirmiya et al. (2006) found no association with specific alleles, but reported significant associations between haplotypes formed from RS1, RS3 and an intronic microsatellite and autism. Taken together, these studies provide convergent, if not conclusive, evidence for a contribution of genetic variation in *AVPR1A* to risk for autism, which is further supported by linkage to the region of the gene (Wassink et al., 2004) and social dysfunction reminiscent of autism found in *avpr1a* knockout mice (Bielsky et al., 2004). Second, regarding oxytocin, genetic variation in the OXTR receptor has been associated with autism in two studies, and the gene itself is located at a suggestive linkage peak for the disorder (Wu et al., 2005). Apart from this genetic evidence, many authors (Modahl et al., 1992; Panksepp, 1993; Insel et al., 1999; Hammock and Young, 2006; Carter, 2007) have speculated about a role for OT in autism. This is plausible, since a core feature of the disease is impaired social interaction and affiliation that are mediated by OT. Several lines of clinical and preclinical evidence support this hypothesis: plasma OT levels were found to be lowered in autism, and correlated with social impairment (Modahl et al., 1998; Green et al., 2001) and autism spectrum disorder patients showed a significant reduction in repetitive behaviours (Hollander et al., 2003) and improvement in social cognition (Hollander et al., 2007) after OT versus placebo infusion.

In addition to these data on autism, the 5′ microsatellite variants of AVPR1A encoding genes also open up a fascinating transspecies perspective on the genetics of social behaviour. Hammock and Young (2005) recently demonstrated that long and short repeat length of a 5′ microsatellite variant that is highly expanded in monogamous, but not promiscuous vole species, predicted individual social behaviour, as well as AVPR1A receptor distribution, in prairie voles (Hammock and Young, 2005). They showed that long and short variants differed in *avpr1a* mRNA expression in transfected cells. These authors also noted that highly homologous microsatellite variants at this locus are found in humans and bonobos (pygmy chimpanzees), both known for high levels of social reciprocity, empathy and sociosexual bonding,

while a 360 bp deletion is found in the common chimpanzee, providing evidence for homologous genetic differences in related highly social and asocial species from different parts of the mammalian lineage. A further link to social behaviour in general is provided by the recent observation of association of RS1–RS3 haplotypes with a personality trait, reward dependence (Knafo et al., 2007). The same group also recently showed an impact of these variants on behaviour during an economic game (the dictator game) and provided post-mortem data indicating that length variation in the RS3 microsatellite had an impact on expression of the AVPR1A mRNA transcript in human hippocampus, providing a cellular mechanism that could link the findings in the vole to those that might operate in humans (Knafo et al., 2007).

The neural mechanism of these genetic associations is presently unknown. However, our previous results strongly suggest that neural circuitry involving amygdala will be involved in these genetic effects. This hypothesis agrees with findings from our group on the neurogenetic architecture of social behaviour in humans. For example, we previously examined what is perhaps the most striking genetic variant of human social behaviour, Williams syndrome (WS), caused by hemizygous micro-deletion of $\sim$25 genes on chromosome 7 (Meyer-Lindenberg et al., 2006a). People with WS are socially fearless, eagerly interacting even with complete strangers and show high empathy (Bellugi et al., 1999). This remarkable hypersociability is coupled with increased non-social anxiety (Dykens, 2003). We studied the neural basis of genetic hypersociability with functional neuroimaging (Meyer-Lindenberg et al., 2005) during viewing of socially relevant (angry and fearful faces) and socially less relevant fearful pictures (dangerous scenes not showing humans). Mirroring the behaviour profile, amygdala activation in participants with WS, relative to matched normal controls, was reduced for threatening faces, but increased for threatening scenes. This indicated a dissociable neural substrate for social and non-social fear, as discussed above in the context of oxytocin. While WS is rare, common variants in the general population impacting on fear and personality traits have also been found to impact on amygdala signalling and regulation (Hariri et al., 2002b; Heinz et al., 2005; Pezawas et al., 2005; Meyer-Lindenberg et al., 2006b; Buckholtz et al., 2007). Based on these data, we have recently used an imaging genetics approach in a large sample of healthy control subjects genotyped for the RS1 and RS3 variants of *AVPR1A*, and for variants (SNPs) in *OXTR* implicated in autism. To probe circuits of emotional arousal, we again employed affectively salient social stimuli (angry and fearful faces) previously shown to reliably activate amygdala and be sensitive to genetic variation in the studies cited above. Results from this approach, submitted for publication, indeed indicate an impact of alleles for polymorphic microsatellite repeats linked to autism in AVPR1A, and SNPs in OXTR, on differential amygdala activation and personality traits in humans. While these results cannot be presented in this review as they have not yet been through peer review, our view of the findings is that they support the proposal that these variants are functionally relevant for brain function related to emotional arousal and social behaviour. Of relevance for our understanding of the molecular architecture of social behaviour in humans, is perhaps the therapeutic promise of this work that is most tantalizing to the clinician: not only do prosocial neuropeptides provide an alternative pathway to treat social dysfunction that is both prominent and disabling to our patients, there is also an, as yet unrealized, possibility for a specific synergy with other salutatory interactions in psychiatry, such as psychotherapy. To further develop these innovative therapies, an understanding of the neural mechanisms through which neuropeptides act in human brain is important and merits extensive further study.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 37

Peptide and non-peptide agonists and antagonists for the vasopressin and oxytocin V_{1a}, V_{1b}, V_2 and OT receptors: research tools and potential therapeutic agents☆

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Abstract: Oxytocin (OT) and vasopressin (AVP) mediate their biological actions by acting on four known receptors: The OT (uterine) and the AVP V_{1a} (vasopressor), V_{1b} (pituitary), V_2 (renal) receptors and a fifth putative AVP V_{1c}? (vasodilating) receptor. This presentation will summarize some highlights of the recent progress, in the design and synthesis of selective peptide agonists, antagonists, radioiodinated ligands, fluorescent ligands and bivalent ligands for these receptors. Here we present published and unpublished pharmacological data on the most widely used agonists, antagonists and labelled ligands. The pharmacological properties of promising new selective OT antagonists and V_{1b} agonists are also presented. This review should serve as a useful guide for the selection of the most appropriate ligand for a given study. The current status of non-peptide OT and AVP antagonists and agonists is also summarized. The relative merits of peptide and non-peptide AVP and OT agonists and antagonists as: (1) research tools and (2) therapeutic agents will be evaluated. Many of the receptor selective peptide agonists and antagonists from this and other laboratories are far more widely used as pharmacological tools for studies on the peripheral and central effects of OT and AVP than their non-peptide counterparts. In addition to OT and to a lesser extent AVP (pitressin), a number of OT and AVP analogues; such as carbetocin (OT agonist) dDAVP (desmopressin, V_2 agonist), terlipressin (V_{1a} agonist), felypressin (V_{1a} agonist) and atosiban (Tractocile OT antagonist) are also in clinical use. Despite much early promise, no non-peptide V_{1a} or OT antagonists are currently in clinical trials. While a number of orally active non-peptide V_2 antagonists (Vaptans); notably, Tolvaptan, Lixivaptan and Satavaptan, are currently in Phase III clinical trials; to date, only the mixed V_2/V_{1a}, antagonist Conivaptan (Vaprisol), has been approved by the US FDA for clinical use (by i.v. administration), for the treatment of euvolemic and hypervolemic hyponatremia

☆Dedicated with gratitude to Dr. Wilbur H. Sawyer, Dr. W.Y. Chan, Dr. Serge Jard and Dr. Claude Barberis.

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DOI: 10.1016/S0079-6123(08)00437-8

in hospitalized patients. Promising new non-peptide V_{1b} and OT antagonists, as well as non-peptide V_2 and OT agonists are now in pre-clinical development.

Keywords: vasopressin; oxytocin; agonist; antagonist; radiolabelled; bivalent; non-peptide; vaptans

Introduction

The past 12 years, have witnessed an extraordinary resurgence of interest in all aspects of neurohypophysial peptide research, including the emergence of a wide variety of more selective agonists and antagonists (both peptide and non-peptide) of the vasopressin (AVP) and oxytocin (OT) receptors. In addition to the numerous publications which have appeared in peer-reviewed journals in the past 12 years, 6 World Congresses on the Neurohypophysial Hormones (WCNH) were held in: (1) Nasu, Japan in 1995 (Saito et al., 1995b), (2) Montreal, Quebec in 1997 (Zingg et al., 1998), (3) Edinburgh, Scotland in 1999 (Douglas et al., 2000), (4) Bordeaux, France in 2001 (Poulain et al., 2002), (5) Kyoto, Japan in 2003 (Kawata and Leng, 2004) and (6) Steamboat Springs, Colorado in 2005. Proceedings from the 2005 WCNH were published in different issues of the Journal of the American Physiological Society. The 7th WCNH was held in Regensburg, Germany in September 2007: (Landgraf and Neumann, 2008). Numerous studies exploring the central and peripheral effects of OT and AVP, using peptides from these laboratories were reported at all seven WCNH's. In addition, during this period, numerous studies, reported in peer reviewed journals have utilized OT and AVP peptide agonists and antagonists, obtained either from the Manning laboratory or from commercial sources (see Pena et al., 2007a for some examples).

OT and AVP receptors belong to a G-protein coupled receptor family characterized by seven putative transmembrane helices. For recent reviews on AVP and OT receptors see Jard, 1998; Barberis et al., 1999; Gimpl and Fahrenholz, 2001; Holmes et al., 2003, 2004. OT receptors (OTRs) are expressed in the uterus, the mammary gland, the ovary, the brain, the kidney, the heart, bone and in endothelial cells (Gimpl and Fahrenholz, 2001). In the uterus, OTRs mediate the uterine contracting (oxytocic) effect of OT (Gimpl and Fahrenholz, 2001). The central effects of OT continue to be the focus of intense investigative scrutiny in animals (Insel and Young, 2001; Bosch et al., 2005; Huber et al., 2005; Ma et al., 2005; Parker et al., 2005; Ring et al., 2006) and in humans (Hollander et al., 2003, 2007; Kirsch et al., 2005), as a possible therapeutic agent for the treatment of autism and other anxiety disorders.

AVP mediates its actions through three known receptors: V_{1a}, V_{1b} and V_2 and a putative vasodilating V_{1c}? receptor. V_{1a} receptors are expressed in the liver, vascular smooth muscle cells, brain and in many other tissues (Jard, 1998; Barberis et al., 1999; Holmes et al., 2003, 2004; Treschan and Peters, 2006). In the vasculature, V_{1a} receptors mediate the pressor actions of AVP by a phospholipase C mediated pathway. In brain, V_{1a} receptors mediate the anxiety (Ma et al., 2005; Ring, 2005) and aggression (Ferris et al., 2006) producing responses to AVP. V_{1b} receptors, present in the anterior pituitary, mediate the ACTH releasing effects of AVP, also by a phospholipase C mediating pathway (Jard, 1998). In a number of publications (Robert et al., 2005), the V_{1b} receptor is also referred to as the V_3 receptor. According to the IUPHAR nomenclature, V_3 is no longer acceptable. Evidence for the presence of V_{1b} receptors in extra-pituitary tissues such as brain, the kidney and the adrenal medulla has also been reported (Saito et al., 1995a, b). Recently the V_{1b} receptor has been shown to mediate anxiety and stress in rats and in humans (Landgraf, 2006). V_2 receptors, present in the collecting duct of the kidney, mediate the antidiuretic action of AVP by an adenylate cyclase mediated pathway (Jard, 1998; Barberis et al., 1999; Holmes et al., 2003, 2004). The pain response to AVP in the rat appears to be modulated by V_2 receptors (Yang et al., 2006). Besides its

antidiuretic and vasoconstrictor properties, AVP can also cause vasodilation (Liard, 1989; Hirsch et al., 1989; Walker et al., 1989; Tagawa et al., 1995; Van Lieburg et al., 1995). The receptor subtype that mediates the vasodilating actions of AVP has to date not been characterized. Studies aimed at doing so have been hampered by the lack of specific vasodilating AVP agonists or antagonists. We recently reported the serendipitous discovery of highly selective AVP hypotensive peptides (Chan et al., 1998; Manning et al., 1999, 2007; Stoev et al., 2006).

Besides their value as pharmacological tools and radioligands, OT and AVP antagonists are of potential clinical value. Antagonists of OT are of potential therapeutic value for the prevention of premature labour (Tsatsaris et al., 2004; Manning et al., 2005; Reinheimer et al., 2005; Stymiest et al., 2005; Akerlund, 2006; Flouret et al., 2006; Tan et al., 2006). Atosiban (d[D-Tyr(Et)^2,Thr^4,Orn^8] vasotocin), a peptide OT antagonist (Melin et al., 1986; Romero et al., 2000; Valenzuela et al., 2000) is the only tocolytic, approved (in Europe) under the tradename Tractocile in, 2001, for the prevention of premature birth (Akerlund, 2006). Non-peptide AVP V_2 antagonists have potential therapeutic value for the treatment of the hyponatremia caused by the syndrome of inappropriate secretion of the antidiuretic hormone (SIADH) (Serradeil-Le Gal et al., 2002b; Hays, 2006; Palm et al., 2006; Schrier et al., 2006; Streefkerk and van Zwieten, 2006; Verbalis, 2006; Cawley, 2007; Chen et al., 2007; Gines, 2007; Munger, 2007; Parashar et al., 2007). The non-selective non-peptide AVP V_2/V_{1a} antagonist, Conivaptan (YM-087) (Tahara et al., 1997, 1998) under the tradename "Vaprisol" was approved for the treatment, by i.v. only, of euvolemic hyponatremia by the FDA in 2005 (Ghali et al., 2006; Verbalis, 2006). Earlier this year, it received FDA approval for the treatment of hypervolemic hyponatremia. Non-peptide V_2 and V_2/V_{1a} antagonists may also have value for the treatment of heart failure (Abraham et al., 2006; Schwarz and Sanghi, 2006). They also have potential as pharmacochaperones for the treatment of X-linked nephrogenic diabetes insipidus (NDI) (Bernier et al., 2006; Robben et al., 2007). Three selective non-peptide V_2 antagonists are currently in clinical trial (Verbalis, 2006): Tolvaptan (OPC 41061) (Yamamura et al., 1998), Satavaptan (SR 121 463) (Serradeil-Le Gal et al., 1996) and Lixivaptan (VPA-985) (Albright et al., 1998; Schrier et al., 2006; Soupart et al., 2006). However none has yet been approved by the FDA. Antagonists of the vascular responses (V_{1a} receptor) to AVP may have clinical potential for the treatment of those patients with hypertension or congestive heart failure (CHF) with concomitant elevated plasma AVP levels (Thibonnier et al., 2001). They may also be of value as "serenics" in the management of anger (Ferris et al., 2006). However, with the exception of the V_{1a} antagonist SRX-251 (Ferris et al., 2006; Guillon et al., 2007a, b), none are currently in clinical trial. Non-peptide AVP V_{1b} antagonists could be of value as diagnostic agents and as therapeutic agents for the treatment of ACTH secreting tumours (Serradeil-Le Gal et al., 2002a, b, 2007) and for treating anxiety and stress (Griebel et al., 2002; Craighead and MacSweeney, 2008).

Scope of the this presentation

This review will focus on documenting the pharmacological properties of the most widely used OT and AVP peptide agonists and antagonists, while also providing data on more promising newer peptides. Space limitations preclude the inclusion of more than the sketchiest details on how these peptides were designed. The original publications and reviews such as (Manning et al., 1981; Hruby and Smith, 1987; Lebl, 1988; Manning and Sawyer, 1989, 1993) should be consulted for more complete descriptions. Other laboratories, most notably, the Lammek, Cordopatis, Fluoret, Lubell, Reinheimer, Slaninova and Stymiest laboratories continue to make important contributions to this field. Unfortunately, space consideration also preclude the inclusion of their work here. For examples of their recent studies see: Prochazka and Slaninova (1995), Belec et al. (2005), Stymiest et al. (2005), Flouret et al. (2006), Fragiadaki et al. (2007), Kowalczyk et al. (2007) and Reinheimer (2007).

Where possible, we will address the issues of species differences (Howl et al., 1991; Ruffolo et al., 1991; Pettibone et al., 1992; Tahara et al., 1999; Andres et al., 2004; Guillon et al., 2004, 2006; Chini and Manning, 2007; Thibonnier et al., 1997) and the lack of single receptor specificity (Manning and Sawyer, 1989, 1993; Chan et al., 2000) of many of these peptides. As noted above, peptide agonists and antagonists have continued to be widely used as pharmacological tools in a wide variety of studies. However, quite a number of studies have utilized some of the earlier non-selective antagonists, clearly unaware that more selective antagonists are available. This applies particularly to OT antagonists. Thus for example, d$(CH_2)_5$[Tyr$(Me)^2$]OVT, which is in fact five times more potent as an AVP V_{1a} antagonist than as an OT antagonist (in vivo) in the rat (Bankowski et al., 1980), continues to be used as a *selective* OT antagonist. Table 15 provides a list of OTAs which are much more selective than d$(CH_2)_5$[Tyr$(Me)^2$]OVT. We have selected for inclusion in the different Tables primarily those peptides which have been used as pharmacological tools in the past and only the most promising of the newer agonists and antagonists which might be useful in the future. Thus Tables 1–3, 5, 6, 8–11, 13–15 provide pharmacological properties in rat bioassays, of a wide variety of AVP and OT agonists and antagonists together with radiolabelled and fluorescent derivatives. However, we do not have rat antioxytocic data for a number of the AVP antagonists in these Tables. We also present preliminary receptor data on bivalent OT and AVP antagonists. In some instances (Tables 4–7, 14–16, 20), we provide human and/or rat receptor binding and functional assay data. All Tables contain a wide variety of old and new AVP V_{1a}, V_{1b}, V_2 and OT receptor ligands for use as potential pharmacological tools in a wide variety of studies. The selective V_{1b} agonists in Tables 4–7 are excellent new tools for probing the human and rat V_{1b} receptors.

In this review we will also attempt to provide a status report on the pre-clinical and clinical development of non-peptide AVP and OT antagonists and on the pre-clinical development of non-peptide AVP and OT agonists. The excellent reviews on non-peptide AVP antagonists (Serradeil-Le Gal et al., 2002b) and on non-peptide OT antagonists (Freidinger and Pettibone, 1997) should be consulted for more in-depth presentations of their chemistry and pharmacology. As noted earlier, after a very slow start, the clinical potential of non-peptide V_2 antagonists is finally beginning to look more promising (Hays, 2006; Verbalis, 2006). We will also review the merits of peptide versus non-peptide AVP and OT agonists and antagonists as (1) research tools and (2) therapeutic agents.

Peptide synthesis

All the OT and AVP agonists, antagonists, radiolabelled and fluorescent ligands from our laboratories were synthesized using the Merrifield solid-phase method (Merrifield, 1963; Stewart and Young, 1984). The procedures used are described in the original publications cited here. For other references see Manning (2008).

Bioassays

Peptides from our laboratories were assayed for agonistic and antagonistic activities in in vitro and in vivo rat oxytocic assays in the rat vasopressor assay and in the rat antidiuretic assay. For agonists, the four-point assay design (Holton, 1948) was used and for antagonists, Schild's pA_2 method (Schild, 1947) was employed. The pA_2 is the negative logarithm of the molar concentration of the antagonist that will reduce the response to 2x units of the agonist to equal the response to 1x unit of the agonist in the absence of antagonist. In practice, this concentration is estimated by finding concentrations above and below the pA_2 dose and interpolating on a logarithmic scale. In the rat in vivo assays, the pA_2 dose (effective dose, ED) is divided by an arbitrarily assumed volume of distribution of 67 ml/kg (Dyckes et al., 1974) in an attempt to derive the approximate molar concentration [M] of the pA_2 dose in the vicinity of the receptors. Thus, in vivo pA_2 values are very rough estimates. The USP Posterior Pituitary

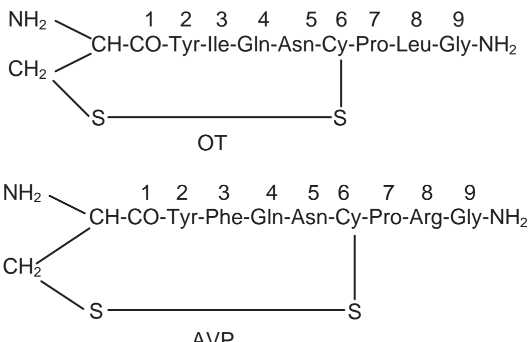
Reference Standard or synthetic OT and arginine–AVP, which had been standardized in oxytocic and vasopressor units against this Standard, were used as agonists for working standards in all bioassays. In vitro oxytocic assays were performed on isolated uteri from diethylstilbestrol-primed rats in a Mg^{2+}-free van Dyke Hasting's solution (Munsick, 1960). In vivo anti-OT potencies were determined in urethane-anesthetized diethylstilbestrol-primed rats as previously described (Chan and Kelly, 1967). Vasopressor assays were performed on urethane-anesthetized and phenoxybenzamine-treated rats as described by Dekanski (1952). The vasodepressor activity of the hypotensive peptides was determined in urethane-anesthetized male rats as previously described (Chan et al., 2001; Manning et al., 2007). Antidiuretic assays were on water-loaded rats under ethanol anaesthesia as described by Sawyer (Sawyer, 1961).

Receptor binding and functional assays

Membranes and/or cell lines which express the rat and human AVP V_{1a}, V_{1b} and V_2 receptors (Birnbaumer et al., 1992; Lolait et al., 1992; Morel et al., 1992; De Keyzer et al., 1994; Sugimoto et al., 1994; Thibonnier et al., 1994) and the human OT receptor (Kimura et al., 1992) were used for binding and functional assays: inositol phosphate accumulation (Bone et al., 1984) for V_{1a}, V_{1b} and OT receptors and cyclic AMP accumulation (Salomon et al., 1974) for V_2 receptors, as previously described (Butlen et al., 1978; Cantau et al., 1980; Andres et al., 2002; Derick et al., 2002; Cheng et al., 2004; Guillon et al., 2006; Pena et al., 2007a, b).

Potent and/or selective oxytocin and vasopressin agonists

OT and AVP have very similar structures and differ only at position 3 and 8 (Tables 1–3). Their structures are as follows:



Because of the similarity of their structures, they exhibit a spectrum of overlapping pharmacological properties in rat bioassays (Tables 1 and 2). Table 1 provides the pharmacological properties of some widely used analogues of OT which

Table 1. Potent and selective agonists for the oxytocin uterine receptor in the rat

No.	Peptide	OT receptor oxytocic (*O*) (U/mg)	V_{1a} receptor vasopressor (*P*) (U/mg)	V_2 receptor antidiuretic (*A*) (U/mg)	Ratios	
					O/*A*	*O*/*P*
	OT[a,b]	520	4	4	130	130
1	[Thr[4]]OT[c]	923	0.4	0.9	1025	2307
2	[HO[1]][Thr[4]]OT[c,d]	4179	4.92	5.3	790	850
3	[Thr[4], Gly[7]]OT[b,c]	166	<0.01	~ 0.002	83,000	$>16,600$
4	[HO[1]][Thr[4], Gly[7]]OT[c]	218	<0.01	0.004	54,500	$>21,800$

Note: Oxytocic activities were assayed on isolated rat uteri suspended in Mg^{2+}-free solution. OT, oxytocin; HO, 1-hydroxy (hydroxyl group replaces α-amino group).

[a]For original reference see Manning et al., 1981.

[b]Affinities for human OT, V_{1a}, V_{1b} and V_2 receptors reported in Barberis et al., 1999; Thibonnier et al., 1997.

[c]Data from Lowbridge et al., 1977.

[d]HO[Thr[4]]OT hOT $K_i = 0.31$ nM; hV_{1a} $K_i = 17$ nM (Barberis and Manning, unpublished).

Table 2. Potent and selective agonists for the vasopressin V_2 receptor in the rat

No.	Peptide	OT receptor oxytocic (O) (U/mg)	V_{1a} receptor vasopressor (P) (U/mg)	V_2 receptor antidiuretic (A) (U/mg)	Ratios	
					A/P	A/O
	AVP[a,b]	14	373	320	0.9	22.8
	AVT ([Ile[3]]AVP)[c]	160	231	127	0.6	0.8
1	[Cha[3]]AVP[c]	0.33	73	294	4	890
2	dDAVP[a,b] (desmopressin)	1.5	0.39	1200	3000	800
3	VDAVP[a]	0.60	0.037	653	17,650	1090
4	dVDAVP[a]	8	Antagonist ($pA_2 = 7.03$)	1230	Infinite	
5	d[Tyr(Me[2])]VDAVP[d]	Antagonist ($pA_2 = 7.38$) (in vivo)	Antagonist ($pA_2 = 7.01$)	1740	Infinite	Infinite

Note: AVP, arginine vasopressin; Tyr(Me), *O*-methyl tyrosine; d, deamino; DAVP, D-Arg[8]VP; V, Val[4]; VT, vasotocin.
[a,b]See corresponding footnotes in Table 1.
[c]Data from Stoev et al., 1999.
[d]Data from Lammek et al., 1989.

Table 3. Potent and selective agonists for the vasopressin V_{1a} receptor in the rat

No.	Peptide	V_{1a} receptor vasopressor (P) (U/mg)	V_2 receptor antidiuretic (A) (U/mg)	OT receptor oxytocic (O) (U/mg)	Ratios	
					P/A	P/O
	AVP[a,b]	373	320	14	1.2	26.6
	LVP ([Lys[8]]VP)[a,c]	270	284	10	0.95	27
1	[Phe[2]]LVP ([felypressin, octapressin)[c]	57	21	0.3	2.7	190
2	OVP ([Orn[8]]VP)[c]	375	92	10	4.1	37.5
3	[Phe[2]]OVP[c]	157	16	0.5	9.8	314
4	LVT ([Ile[3]]LVP)[c]	130	24	78	5.4	1.7
5	[Phe[2]]LVT[c]	32	1	1	32	32
6	OVT([Ile[3], Orn[8]]VP)[c]	104	2.5	42	41.6	2.5
7	[Phe[2]]OVT[a,b]	124	0.55	1	225	124
8	F-180[d]	164	0.19		863	

Note: F-180, Hmp–Phe–Ile–Hgn–Asn–Cys–Pro–Dab(Abu)–Gly–NH_2; Hmp, 2-hydroxy-3-mercaptopropionic acid; Hgn, homoglutamine; Dab, 2,4-diaminobutyric acid; Abu, 2-aminobutyric acid.
[a,b]See corresponding footnotes in Table 1.
[c]Data from Berde and Boissonnas, 1968.
[d]Data (biological potency values are in IU/μmol) from Bernadich et al. (1998). For original synthesis and biological activity data see Aurell et al., 1991.

exhibit greater potency and/or selectivity than OT in the oxytocic, antidiuretic and/or vasopressor rat bioassays. Thus [Thr[4], Gly[7]]OT and HO[Thr[4], Gly[7]]OT which exhibit negligible vasopressor and antidiuretic activities are highly selective agonists for the rat uterine receptor. However, as an example of profound species differences, [Thr[4], Gly[7]]OT is not highly selective for the human OT receptor (Barberis et al., 1999; Guillon et al., 2004; Chini and Manning, 2007). HO[1] [Thr[4]]OT exhibits an eightfold increase in oxytocic activity relative to OT in the rat and is thus one of the most potent OT agonists reported to date. In the human receptor binding assay, HO[Thr[4]]OT exhibits high affinity and selectivity for the human OTR (hOTR) ($K_i = 0.31$ nM) with respect to the $hV_{1a}R$ ($K_i = 17$ nM) (Dr. Claude Barberis, personal communication). Thus HO[Thr[4]]OT could serve as a very useful lead for the design and development of OT analogues having protracted activity relative

to OT, for the possible treatment of autism (Hollander et al., 2003, 2007) and for the prevention of postpartum haemorrhage (Su et al., 2007). Table 2 lists a number of AVP analogues which exhibit enhanced potency and selectivity in the rat antidiuretic assay with respect to their vasopressor and oxytocic activities. In rat bioassays and in human receptor binding assays dVDAVP (Manning et al., 1973; Barberis et al., 1999) is a more potent and selective antidiuretic agonist than the widely used dDAVP (Zaoral et al., 1967). As shown in Table 4, dDAVP has a higher affinity for the human V_{1b} receptor than for the human V_2 receptor. DDAVP had previously been reported to be a V_{1b} agonist in humans (Saito et al., 1997). In fact, as another example of species differences, while dDAVP is selective for the rat V_2 receptor with respect to the rat V_{1a} and V_{1b} receptors, it is not selective for the human V_2 receptor with respect to the V_{1a} and V_{1b} receptors (Barberis et al., 1999; Guillon et al., 2004; Chini and Manning, 2007). The search for a V_2 agonist which is selective for the human V_2 receptor with respect to the V_{1a}, V_{1b} and OT receptors is still a challenging goal in this field. Table 3 lists AVP analogues which exhibit selective vasopressor agonism with respect to antidiuretic and oxytocic activities in rat bioassays. Peptide 7, Table 3, [Phe^2]OVT (Huguenin, 1964) has been widely used as a selective V_{1a} receptor agonist (Sawyer and Manning, 1988). However, binding assays indicate that this peptide is not selective for the rat V_{1a} receptor (Guillon, personal communication). F-180, synthesized at Ferring (Aurell et al., 1991) is even more selective than [Phe^2]OVT in rat bioassays (Table 3). However, to date it has not been widely used as a selective V_{1a} agonist. In human receptor assays (Andres et al., 2002), F-180 has been shown to be a highly selective V_{1a} agonist. However, by contrast with its in vivo V_{1a} receptor selectivity in the rat, in rat receptor binding and functional assays, F-180 is not a selective V_{1a} agonist (Andres et al., 2002). F-180 is clearly a powerful new tool for the study of V_{1a} receptors in humans. As a highly selective vasoconstrictor, it is a potential new treatment for bleeding oesophageal varices in humans (Bernadich et al., 1998).

V_{1b} receptor agonist development

Selective agonists for human and rat V_{1b} receptors

Following the historic original synthesis of OT and AVP in the du Vigneaud laboratory (du Vigneaud et al., 1954a, b), selective agonists for the AVP V_{1a} and V_2 receptors were uncovered relatively easily in the mid to late sixties, by classical structure activity studies (for reviews see Berde and Boissonnas, 1968; Hruby and Smith, 1987; Jost et al., 1988; Manning and Sawyer, 1989, 1993) (Tables 4–7). For a variety of reasons, the search for selective agonists for the rat and human V_{1b} receptors proved to be much more challenging and elusive. The lack of a routine bioassay for the ACTH releasing effects of AVP was one of the main reasons for the delay in uncovering selective V_{1b} agonists. d[D-3-Pal^2]AVP was reported to be a selective V_{1b} agonist for the sheep V_{1b} receptor (Schwartz et al., 1991). It is commercially available and has been utilized in a number of studies as a selective probe of V_{1b} receptors. However, it was subsequently shown not to exhibit selective V_{1b} agonism for the human V_{1b} receptor (Barberis et al., 1999) (Table 4) or for the rat V_{1b} receptor (Derick et al., 2002) (Table 5). The discovery of the V_{1b} receptor (Antoni, 1984; Jard, 1986) and the subsequent cloning of the V_{1b} receptor (de Keyzer et al., 1994; Sugimoto et al., 1994; Saito et al., 1995a), together with the development of V_{1b} receptor binding and functional assays, greatly facilitated the recent discovery of AVP agonists which are selective for (a) the human V_{1b} receptor (Derick et al., 2002; Cheng et al., 2004; Guillon et al., 2006) (Tables 4 and 5), (b) the rat V_{1b} receptor (Pena et al., 2007a, b) (Table 6) and (c) which exhibit selectivity for both the human and the rat V_{1b} receptors (Table 7) (Guillon et al., 2007b).

Modification of dAVP at position 4 led to first selective human V_{1b} receptor agonists

Table 4 provides the human V_{1b}, V_{1a}, V_2 and OT receptor affinities for five position 4 analogues of 1-deamino-arginine vasopressin (dAVP) (Huguenin and Boissonnas, 1966) together with those of other

widely used AVP agonists. d[Cha^4]AVP, is the first specific agonist for the human V_{1b} receptor (Derick et al., 2002). Further modifications of position 4 in dAVP uncovered four new peptides which are even more selective for the human V_{1b} receptor than d[Cha^4]AVP. These are analogues of dAVP which have the Gln^4 residue replaced by Leu, Orn, Lys and homoarginine (Har) (Cheng et al., 2004). d[Cha^4]AVP, has been used as a highly valuable pharmacological tool in a variety of important studies (Andres et al., 2004; Derick et al., 2004; Huber et al., 2005; Griffante et al., 2005; Reymond-Marron et al., 2005, 2006; Volpi et al., 2006; Rodrigo et al., 2007). However, as noted below, as yet another example of species differences, d[Cha^4]AVP while fully selective for the human V_{1b} receptor, with respect to the hV_{1a}, hV_2 and hOT receptors is not selective for the rat V_{1b} receptor with respect to the rat V_2 receptor (Tables 5 and 6).

Table 4. Position 4 analogues of 1-deamino-arginine vasopressin (dAVP) (peptides 1–5) which exhibit high affinities and selectivities for the human V_{1b} receptor[a]

No.	Peptide	Affinity (K_i) (nM)			
		hV_{1b}R	hV_2R	hV_{1a}R	hOTR
	AVP[b]	0.68	1.2	1.1	1.7
	dAVP[b]	0.37	5.0	3.8	–
	dDAVP[c]	5.8	23.3	62.4[c]	–
	d[Val^4]AVP[d]	0.29	1.2	11.4	136
	dVDAVP[d]	24.5	0.8	–	–
	d[D-3-Pal^2]AVP[b]	13.8	9600	34.2	–
1	d[Cha^4]AVP[d]	1.2	750	151	240
2	d[Leu^4]AVP[d]	0.23	245	44.1	211
3	d[Orn^4]AVP[d]	0.49	1125	88	326
4	d[Lys^4]AVP[d]	1.8	11,170	463	1429
5	d[Har^4]AVP[d]	0.52	1386	48.2	1364

[a]Binding assays were performed on plasma membranes from CHO and AtT-20 cells stably transfected with the human VP/OT receptors.
[b]Data from Derick et al., 2002.
[c]Data from Saito et al., 1997.
[d]Data from Cheng et al., 2004.

Profound species differences: selective human V_{1b} agonists exhibit potent antidiuretic activities in rat bioassays

As noted above, modifications of dAVP at position 4, led to the breakthrough discovery of agonists which are selective for the human V_{1b} receptor (Table 4). In a follow-up study, we examined the pharmacological properties of these four peptides: d[X^4]AVP (where X = Leu, Orn, Lys and Har), in rat bioassays and in rat receptor assays

Table 5. Selective human V_{1b} agonists are non-selective V_{1b} agonists in the rat[a]

No.	Peptide	Rat antidiuretic activity (U/mg)	Affinity (K_i) (nM)			
			rV_{1b}R	rV_2R	rV_{1a}R	rOTR
	AVP[b]	323[g]	0.29	0.45	2.6	1.7
	dAVP[c]	1745[f]	0.20	0.76	10.8	0.97
	dDAVP[d]	1200[f]	9.29	0.3	100	–
	d[Val^4]AVP[e]	1150[f]	0.25	0.3	60	n.d.
	dVDAVP[b]	1230[f]		0.3	316	–
	d[D-3-Pal^2]AVP[d]	1	112	69	450	–
1	d[Cha^4]AVP[e]	133.6	1.40	12.7	2297	1430
2	d[Leu^4]AVP[d]	378	0.04	3.1	1252	481
3	d[Orn^4]AVP[d]	260.3	0.45	3.4	900	997
4	d[Lys^4]AVP[d]	34.8	9.8	24.6	1478	5042
5	d[Har^4]AVP[d]	504.8	0.32	0.6	32	2996

[a]See Table 2, footnote a.
[b]Data from Guillon et al., 2007b.
[c]Data from Cheng et al., 2004.
[d]Data from Guillon et al., 2006.
[e]Data from Derick et al., 2002.
[f]Data from Sawyer et al., 1974.
[g]Data from Manning et al., 1976.

(Guillon et al., 2006) (Table 5). The affinities of these position-4 analogues for the rat and human V_{1b}, V_{1a} and OT receptors are almost identical. These peptides exhibit high affinities for the rat and human V_{1b} receptors and low affinities for the rat and human V_{1a} and OT receptors. However their affinities for the rat and human V_2 receptors are strikingly different. Whereas these peptides have low affinities for the human V_2 receptor, by contrast, they exhibit high affinities for the rat V_2 receptor. They also exhibit potent antidiuretic agonism in rat bioassays (Table 5) (Guillon et al., 2006). These findings present further evidence for profound species differences between the human and rat V_2 receptors. The findings in Tables 4 and 5 raise important questions regarding the unwanted V_{1b} agonism of therapeutically used antidiuretic agonists, such as dDAVP (Saito et al., 1997). These findings also point to the need for caution when describing peptides with high antidiuretic activities and low vasopressor activities as "selective" V_2 agonists. Since all of the potent antidiuretic agonists in Table 5 are potent V_{1b} agonists also, they should not be referred to as "selective" V_2 agonists with respect to all VP receptors. Thus the design of highly potent V_2 agonists which are devoid of V_{1b} receptor agonism in the rat and in humans remains a challenging goal. As noted below, these findings also provided very important leads to the design of the first selective V_{1b} agonists in the rat (Pena et al., 2007a, b).

First selective agonists for the rat V_{1b} receptor

Although AVP was synthesized in 1954 (du Vigneaud et al., 1954b) it was not until 1984 that its role in the release of ACTH and thus in stress was found to be mediated by a receptor other than the well-known V_{1a} and V_2 receptors (Antoni, 1984; Jard et al., 1986). This receptor was referred to as the V_{1b} receptor. The cloning of the rat V_{1b} receptor (Saito et al., 1995a, b) greatly facilitated the design of selective V_{1b} agonists in the rat. We very recently reported a breakthrough in the 20 year search for selective agonists for the rat V_{1b} receptor (Pena et al., 2007a, b). This breakthrough was based on leads uncovered in our successful design of selective agonists for the human V_{1b} receptor: d[Cha^4]AVP, d[Leu^4]AVP, (Tables 4 and 5) (Derick et al., 2002; Cheng et al., 2004) noted above. Modifications of these two peptides at position 8 with Lys, diaminobutyric acid (Dab) and diaminopropionic acid (Dap) led to four peptides which exhibit high affinities and selectivities for the rat V_{1b} receptor (Table 6). In functional tests, they are full V_{1b} agonists. These selective rat V_{1b} agonists are excellent new research tools for exploring the role of AVP in mediating the release of ACTH and for studies on the role of AVP in stress. Remarkably, one of these selective V_{1b} agonists, d[Leu^4,Lys^8]VP (Pena et al., 2007b), had been reported over 30 years ago (Dyckes et al., 1973), 11 years before the discovery of the AVP V_{1b} receptor. Because it exhibited very weak activities in the standard vasopressor and antidiuretic rat bioassays, it merited very little interest, until it reemerged in our studies as a potent and selective V_{1b} agonist in the rat (Pena et al., 2007a, b). An editorial accompanying the Pena et al., 2007b publication, lauds d[Leu4, Lys8]VP as "a promising tool for the study of key aspects of V_{1b} receptor function" (Arban, 2007). Besides their value as research tools, these V_{1b} agonists provide useful leads to the design of selective antagonists, radioiodinated, fluorescent and bivalent ligands for the human and rat AVP V_{1b} receptors.

Selective agonists for the rat V_{1b} receptor are also selective agonists for the human V_{1b} receptor

The four peptides in Table 6 namely, d[Cha^4, Lys^8]VP, d[Cha^4, Dab^8]VP, d[Leu^4, Lys^8]VP and d[Leu^4, Dap^8]VP which are selective agonists for the rat V_{1b} receptor were subsequently found to be potent and selective agonists for the human V_{1b} receptor (Guillon et al., 2007b) (Table 7). So all four of these peptides could be used as V_{1b} receptor probes in studies in both rats and human.

Selective agonists for the putative vasopressin vasodilating receptor

Table 8 contains a series of novel selective AVP hypotensive agonists. These are a new class of

Table 6. Position 8 analogues of d[Cha[4]]AVP (A) and d[Leu[4]]AVP (B) exhibit drastic reductions in antidiuretic activities, high affinities and selectivities for the rat V_{1b} receptor[a]

No.	Peptide	Rat antidiuretic activity (U/mg)	Affinity (K_i) (nM)			
			$rV_{1b}R$	rV_2R	$rV_{1a}R$	rOTR
	AVP[b]	323[e]	0.29	0.45	2.6	1.7
A	d[Cha[4]]AVP[c]	133.6	1.40[b]	12.7	2297[b]	1430
1	d[Cha[4], Lys[8]]VP[b]	0.82	1.88	596	9093	586
2	d[Cha[4], Dab[8]]VP[b]	1.04	0.79	447	4378	432
B	d[Leu[4]]AVP[d]	378	0.04	3.1	1252	481
3	d[Leu[4], Lys[8]]VP[b,f]	10.51	0.16	101	3786	64
4	d[Leu[4], Dap[8]]VP[b]	0.75	0.38	237	3269	134

[a]See Table 2, footnote a.
[b]Data from Pena et al., 2007a.
[c]Data from Derick et al., 2002.
[d]Data from Guillon et al., 2006.
[e]Data from Manning et al., 1976.
[f]Original synthesis reported in Dyckes et al., 1973.

Table 7. Position 8 analogues of d[Cha[4]]AVP (A) and d[Leu[4]]AVP (B) exhibit high affinities and selectivities for both human and rat V_{1b} receptors[d]

No.	Peptide	Affinity (K_i) (nM)							
		$rV_{1b}R$	$hV_{1b}R$	rV_2R	hV_2R	$rV_{1a}R$	$hV_{1a}R$	rOTR	hOTR
	AVP[a]	0.29	0.68	0.45	1.2	2.6	1.1	1.7	1.7
	dAVP[b]	0.20[a]	0.37	0.76	5.0	10.8[a]	3.8	0.97	–
A	d[Cha[4]]AVP[b]	1.40[a]	1.2	12.7	750	2297[a]	151	1430	240
1	d[Cha[4], Lys[8]]VP[a]	1.88	2.2[e]	596	11,484[e]	9093	283[e]	586	141[e]
2	d[Cha[4], Dab[8]]VP[a]	0.79	0.52[e]	447	7337[e]	4378	176[e]	432	349[e]
B	d[Leu[4]]AVP[c]	0.04	0.23	3.1	245	1252	44.1	481	211
3	d[Leu[4], Lys[8]]VP[a]	0.16	0.51[e]	101	6713[e]	3786	69.3[e]	64	29[e]
4	d[Leu[4], Dap[8]]VP[a]	0.38	0.60[e]	237	8730[e]	3269	91.7[e]	134	83[e]

[a–c]See corresponding footnote in Table 4.
[d]See Table 2, footnote a.
[e]Data from Guillon et al., 2007b.

Table 8. Selective vasopressin hypotensive agonists in the rat

No.	Peptide	Vasodepressor ED (μg/100 g)[a,b]
A	$d(CH_2)_5$[D-Tyr(Et)[2], Arg[3], Val[4]]AVP[c]	4.66
B	$d(CH_2)_5$[D-Tyr(Et)[2], Arg[3], Val[4], Arg[7], Eda[9]]AVP[c]	1.10
C	$d(CH_2)_5$[D-Tyr(Et)[2], Arg[3], Val[4], Lys[7], Eda[9]]LVP[d]	0.53
1	$d(CH_2)_5$[D-Tyr(Bu^t)[2], Arg[3], Val[4], Lys[7], Eda[9]]LVP[d]	0.33
2	$d(CH_2)_5$[D-Tyr(Pr^n)[2], Arg[3], Val[4], Lys[7], Eda[9]]LVP[d]	0.18
3	$d(CH_2)_5$[D-Tyr(Pr^i)[2], Arg[3], Val[4], Lys[7], Eda[9]]LVP[d]	0.15
4	$d(CH_2)_5$[D-Tyr(Pr^i)[2], Arg[3], Val[4], Lys[7], Eda[9]←Tyr[10]]LVP[d]	0.14

[a]ED, effective dose (in μg 100/g i.v.) is the dose that produces a vasodepressor response of 5 cm^2 AUC in the 5 min period following injection of test peptide. AUC, area under the vasodepressor response curve (see Chan et al., 2001, for details of vasodepressor assays).
[b]All peptides exhibited undetectable or negligible agonistic or antagonistic activities in the standard antidiuretic, vasopressor and oxytocic (in vitro, no Mg^{2+}) assays.
[c]Data from Chan et al., 2001.
[d]Data from Stoev et al., 2006.

AVP agonists which appear to lower blood pressure in the rat, by acting on the heretofore uncharacterized putative AVP vasodilating receptor. This new class of AVP agonists was discovered serendipitously during the course of studies on position 3 in one of our potent AVP V_2/V_{1a}/OT antagonists $d(CH_2)_5$[D-Tyr(Et)^2, Val^4]AVP (A) (Manning et al., 1982). Peptide (B), ($d(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4]AVP, the Arg^3 analogue of (A), exhibits a profound hypotensive effect and is devoid of the characteristic vasopressor, antidiuretic and oxytocic agonistic or antagonistic effects of AVP agonists and antagonist (Chan et al., 1998). Peptide (B) exhibits a hypotensive ED = 4.66 μg/100 g [ED is the dose (in μg/100 g i.v.) that produces a vasodepressor response of 5 cm^2 AUC in the 5-min period following injection of the test peptide (AUC, area under the curve)] (Chan et al., 2001). Peptide (C), $d(CH_2)_5$[D-Tyr(Et)^2, Arg^3, Val^4, Lys^7, Eda^9]LVP the Lys^7, Lys^8, Eda^9 analogue of (B) (Eda = ethylenediamine), was modified at positions 2, 6 and 9. All of the resulting new peptides exhibit significant gains in hypotensive potency relative to the parent hypotensive peptide (B). Two in particular, the D-Tyr(Pr^n)^2 (where Pr^n = normal propyl) and the D-Tyr (Pr^i)^2 (where Pr^i = isopropyl) analogue of C are 26 and 31 times more potent than (B) (Stoev et al., 2006). They have served as promising leads to new hypotensive peptides (Manning et al., 2007). Some of these selective vasodepressor agonists may (1) be helpful in the search for the aforementioned putative AVP vasodilatory receptor; (2) be useful as new pharmacological tools in studies on the cardiovascular role(s) of AVP; and (3) lead to the development of members of a new class of antihypertensive agents. The hypotensive peptides (B) and (C) have been utilized respectively in two elegant studies by Yu et al. (2003) and by Tabrizchi and Ford (2004). While we have succeeded in synthesizing a number of radioiodinateable ligands with potent hypotensive activity, their ^{125}I derivatives exhibit significant non-specific binding. The challenge remains to design ligands with higher affinities, in the hope of diminishing non-specific binding. Such high affinity, high specific ligands are needed to help to localize the putative vasodilating VP receptor.

Potent cyclic and linear AVP V_{1a} receptor antagonists

Table 9 lists a number of highly potent AVP V_{1a} receptor antagonists. All of these are highly selective with respect to the V_2 receptor. However, it should be noted that the widely used V_{1a} antagonist $d(CH_2)_5$[Tyr(Me)^2]AVP (Kruszynski et al., 1980) (No. 1, Tables 9 and 10), also referred to in many publications as Manning Compound is also a potent OT antagonist in vitro and furthermore, it exhibits OT antagonism (in vivo) (Kruszynski et al., 1980; Chan et al., 1996) (Table 10). $d(CH_2)_5$[Tyr(Me)^2]AVP is also a mixed V_{1a}/OT antagonist for human VP and OT receptors (Barberis et al., 1999). With the exception of peptides 1–3, Tables 9 and 10, none of the V_{1a} antagonists in Table 9 have been examined in in vitro or in vivo rat oxytocic assays. However, it is quite likely that they will all exhibit OT antagonism. So caution should be used in interpreting data from studies which seek to discriminate between V_{1a} and OT receptors with any of these peptides. For such studies, the highly selective V_{1a} antagonist $d(CH_2)_5$ [Tyr(Me)^2, Dab^5]AVP (No. 3, Table 10) (Chan et al., 1996), which is devoid of OT and V_2 agonism and antagonism, although possessing substantially reduced anti-V_{1a} potency, offers clear advantages over any of the other V_{1a} antagonists in Table 9. It has been used as a highly selective probe of AVP V_{1a} receptors (Miller et al., 2002). A number of the linear V_{1a} antagonists or closely related analogues in Table 9 (Manning et al., 1988, 1990, 1991, 1992a) have served as precurors for radioiodination, biotinylation and fluorescent labelling (see Howl et al., 1993; Thibonnier et al., 1993; Barberis et al., 1995; Strakova et al., 1997; Durroux et al., 1999). In fact, the radioiodinated derivatives of peptide 9 (Table 9) i.e. ^{125}I[HO]LVA, which exerts a high affinity for both rat and human V_{1a} receptors (Barberis et al., 1995, 1999) has been cited in hundreds of studies as a selective radioligand for V_{1a} receptors.

Table 9. Potent and selective, with respect to V_2 receptor but not OT receptor, cyclic and linear antagonists of the vasopressin V_{1a} receptor in the rat

No.	Peptide	Antivasopressor		Antiantidiuretic		ED ratio[c]
		ED[a]	pA_2[b]	ED[a]	pA_2[b]	
1	$d(CH_2)_5[Tyr(Me)^2]AVP$[d] (Manning compound)	0.16	8.62	Agonist (0.31 U/mg)		
2	$d(CH_2)_5[Tyr(Me)^2$, $Dap^5]AVP$[e]	2.16	7.49	ND		
3	$d(CH_2)_5[Tyr(Me)^2$, $Dab^5]AVP$[e]	12.9	6.71	ND		
4	$d(CH_2)_5[Tyr(Me)^2$, Ala–$NH_2^9]AVP$[f]	0.13	8.75	Mixed ~ 77	~ 6.0	~ 590
5	$dP[Tyr(Me)^2$, Arg–$NH_2^9]AVP$[f]	0.21	8.52	0.083		
6	Phaa–D-Tyr(Et)–Phe–Gln–Asn–Lys–Pro–Arg–NH_2[g]	0.07	9.05	Mixed ~ 40	~ 6.3	~ 570
7	Phaa–D-Tyr(Et)–Phe–Gln–Asn–Lys–Pro–Arg–Tyr–NH_2[h,i]	0.15	8.64	~ 0.001 U/mg		
8	LVA (Ph Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Arg –Tyr–NH_2[j]	0.08	8.94	Agonist (0.042 U/mg)		
9	4-HO–Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Arg —NH_2 (HO–LVA)[k,l]		8.47	0.056 U/mg		
10	Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Arg–Lys–NH_2[m]					

Note: Convenient derivatization of the Lys^9 ε-amino group of No. 10 with aminomethylcoumarin or biotin leads to highly selective V_{1a}-ligands that are useful for receptor localization and purification studies (Howl et al., 1993). Human receptor affinity data for many of these peptides are reported in Thibonnier et al., 1997.

[a]The ED is defined as the dose (in nmol/kg) that reduces the response to 2x units of agonist to equal the response to 1x unit.

[b]Estimated in vivo pA_2 values represent the negative logarithms of the EDs divided by the estimated volume of distribution (67 ml/kg) (Dyckes et al., 1974).

[c]ED ratio: antiantidiuretic ED/antivasopressor ED.

[d]Data from Kruszynski et al., 1980.

[e]Data from Chan et al., 1996.

[f]Data from Manning et al., 1992b.

[g]Data from Manning et al., 1990.

[h]Data from Manning et al., 1991.

[i]Also used to prepare radioiodinated ligand by Strakova et al., 1997.

[j]Data from Manning et al., 1988.

[k]Data from Manning et al., 1992a.

[l]Rat and human receptor affinity data for HO–LVA and $[^{125}I]$HO–LVA reported, respectively, in Barberis et al., 1995, 1999.

[m]This linear AVP analogue is an extremely high-affinity and selective antagonist at rat V_{1a}-vasopressin receptors. ($V_{1a}K_i = 0.09$ nM; $V_2K_i = 59$ nM; selectivity = 656) (Howl et al., 1993).

Table 10. Modifications of V_{1a} antagonist $d(CH_2)_5[Tyr(Me)^2]AVP$ which reduce or eliminate its in vitro and in vivo anti-OT potency in the rat

No.	Peptide	Anti-OT (in vitro)	Anti-OT (in vivo)	Anti-V_{1a} (in vivo)	Anti-V_{1a}/anti-OT selectivity
		pA_2[a]	pA_2[b]	pA_2[b]	
1	$d(CH_2)_5[Tyr(Me)^2]AVP$[c] (Manning compound)	8.13	6.62	8.67	100
2	$d(CH_2)_5[Tyr(Me)^2$, $Dap^5]AVP$[d]	5.83	ND	7.49	Infinite
3	$d(CH_2)_5[Tyr(Me)^2$, $Dab^5]AVP$[d]	ND[e]	ND	6.71	Infinite

[a]In vitro pA_2 values represent the negative logarithm to the base 10 of the average molar concentration [M] of the antagonist that reduces the response to 2x units of agonist to the equal the response seen with 1x units of agonist administered in the absence of the antagonist.

[b]In vivo pA_2 values are estimates since the molar concentration for the in vivo pA_2 is estimated by dividing ED by the estimated volume of distribution of (67 ml/kg) (Dyckes et al., 1974). ED is defined as the dose (nmol/kg intravenously) of the antagonist that reduces the response to 2x units of agonist to the response with 1x units of agonist administered in the absence of the antagonist.

[c]Data from Kruszynski et al., 1980.

[d]Data from Chan et al., 1996.

[e]ND, not detectable (weak agonist, <0.03 U/mg).

Non-selective rat and human V_{1b}/V_{1a} antagonists

To date, there are no selective peptide V_{1b} antagonists. However, as shown in Tables 11 and 12, a number of cyclic and linear V_{1a} antagonists have been shown to exhibit VP V_{1b} antagonism for rat and/or human receptors in vitro and in vivo. A recent study by Ma et al. (2005) reported that the cyclic V_{1b}/V_{1a} antagonist dP[Tyr(Me)^2, Arg–NH$_2^9$]AVP (Manning et al., 1992b) blocked the ACTH releasing effects of AVP in vivo in the rat. This peptide was also recently reported (Serradeil-Le Gal et al., 2002b) to be a human receptor V_{1b} antagonist ($K_i = 21$ nM). A number of potent linear (rat and human) V_{1a} antagonists have also been reported to exhibit somewhat weaker antagonism for the human V_{1b} receptor. A very recent study utilized the linear V_{1b}/V_{1a} antagonist, Phaa–D-Tyr(Et)–Phe–Gln–Asn–Lys–Pro–Arg–NH$_2$ (No. 3, Table 12) (Thibonnier et al., 1997) to block the ACTH releasing effects of AVP in vivo in the rat (Subburaju and Aguilera, 2007).

Cyclic and linear V_2/V_{1a} antagonists for rat VP receptors

Table 13 lists non-selective and selective cyclic and linear AVP V_2/V_{1a} antagonists in the rat: Peptides 1–5 are cyclic and Peptides 6–10 are linear (Manning and Sawyer, 1989, 1993). Peptides 3–5 are selective for V_2 versus V_{1a} receptors. Thus d(CH$_2$)$_5$[D-Ile^4, Ile^4]AVP, with an ED Ratio of 39, desGly–NH$_2$, d(CH$_2$)$_5$[D-Ile^2, Ile^4]AVP, with an ED ratio of 400 and d(CH$_2$)$_5$[D-Ile^2, Ile^4, Ala–NH$_2^9$]AVP, with an ED ratio of 83 have all been utilized in a wide variety of studies, as selective probes for AVP V_2 receptors. A few of these peptides and their analogues have been shown to exhibit oxytocic antagonism in the rat (Manning et al., 1987). However, we do not have antioxytocic rat data on all of these. Thus, while some are selective V_2 antagonists with respect to AVP V_{1a} receptors in the rat, it is highly probable that none are selective with respect to the rat OT receptor. So these V_2 antagonists should not be used to discriminate between V_2 and OT receptors in the rat.

Table 11. Non-selective antagonists of rat vasopressin V_{1b}/V_{1a} receptors

No.	Peptide	Antivasopressor (anti-V_{1a}) pA_2[a]	Antidiuretic activity (U/mg)	Anti-ACTH release (anti-V_{1b}) K_i (nM)	Anti OT in vitro pA_2
1	dP[Tyr(Me)^2]AVP[b]	7.96	3.5	Rat = 3.3[d]	7.61[b]
2	dP[Tyr(Me)^2, Arg–NH$_2^9$]AVP[c]	8.52	0.083	Human = 21[e,f]	

[a]Estimated in vivo pA_2 values represent the negative logarithms of the EDs divided by the estimated volume of distribution (67 ml/kg).
[b]Data from Manning and Sawyer (1993).
[c]Data from Manning et al., 1992a.
[d]Data from Antoni (1984).
[e]Data from Serradeil-Le Gal et al., 2002b.
[f]Reported by Ma et al., 2005 to block the AVP-stimulated release of ACTH in vivo in the rat.

Table 12. Affinities (nM) of non-selective antagonists of human vasopressin V_{1b}/V_{1a} receptors

No.	Peptide	hV_{1b}R	hV_{1a}R	hV_2R	hOTR
1	Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Arg–Tyr–NH$_2$ (LVA)[a]	9.4	0.8	282	1.1
2	4-HO–Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Arg–NH$_2$ (HO–LVA)[a]	5.9	0.08	137	2.7
3	Phaa–D-Tyr(Et)–Phe–Gln–Asn–Lys–Pro–Arg–NH$_2$[b]	31	0.8	302	–

[a]Data and original references from Durroux et al., 1999.
[b]Data from Thibonnier et al., 1993, 1997.

Table 13. Non-selective and selective cyclic and linear V_2/V_{1a} antagonists for rat receptors

No.	Peptide	Antiantidiuretic (A) (anti-V_2)		Antivasopressor (P) (anti-V_{1a})		ED ratio[c] (A/P)
		ED[a]	pA_2^b	ED[a]	pA_2^b	
1	$d(CH_2)_5$[D-Tyr(Et)^2]VAVP[d]	1.1	7.81	0.45	8.22	0.4
2	desGly,$d(CH_2)_5$[D-Tyr(Et)^2, Val^4]AVP[d]	1.5	7.69	0.45	8.17	0.3
3	$d(CH_2)_5$[D-Ile^2, Ile^4]AVP[d]	0.67	8.04	26	6.42	39
4	desGly–NH_2,$d(CH_2)_5$[D-Ile^2, Ile^4]AVP[d]	0.90	7.88	~400	~5.2	~440
5	$d(CH_2)_5$ [D-Ile^2, Ile^4, Ala–NH_2^9]AVP[d]	0.46	8.16	38	6.25	83
6	Aaa–D-Tyr(Et)–Phe–Val–Asn–Abu–Pro–Arg–Arg–NH_2[d]	0.53	8.11	1.2	7.75	2.3
7	Phaa–D-Tyr(Et)–Phe–Val–Asn–Abu–Pro–Arg–Arg–NH_2[d]	0.69	7.99	0.26	8.42	0.4
8	*t*-Baa–D-Tyr(Et)–Phe–Val–Asn–Abu–Pro–Arg–Arg–NH_2[d]	0.83	7.91	0.71	7.99	0.9
9	Pa–D-Tyr(Et)–Phe–Val–Asn–Abu–Pro–Arg–Arg–NH_2[e]	1.3	7.74	14	6.71	11
10	Pa–D-Tyr(Et)–Phe–Val–Asn–Nva–Pro–Arg–Arg–NH_2[e]	0.60	8.07	13	6.73	22

Note: Aaa, adamantaneacetyl; Phaa, phenylacetyl; *t*-Baa, *tert*-butylacetyl; Pa, propionyl; Nva, norvaline. The in vivo antioxytocic (data from Manning et al., 1987) pA_2 values of peptides 1–3 are as follows: No.1 = 7.47; No.2 = 6.98; No.3 = 6.90.

[a,b,c]See corresponding footnotes of Table 7.

[d]Data and original references from Manning and Sawyer (1989).

[e]Data and original references from Manning and Sawyer (1993). Human receptor affinity data for many of these peptides are reported in Thibonnier et al., 1997.

Table 14. Rat anti-V_2 data and human V_2 receptor affinities of AVP V_2/V_{1a} antagonists

No.	Peptide	Anti-V_2 (rat) pA_2[b,c]	K_i[a] (nM) (hV_2R)
1	$d(CH_2)_5$[D-Tyr(Et)^2, Tic^3]VAVP	7.69	13.5
2	$d(CH_2)_5$[D-Tyr(Et)^2, Pro^3]VAVP	7.77	0.5
3	$d(CH_2)_5$[D-Tyr(Et)^2, Tyr^3]VAVP	7.58	96
4	$d(CH_2)_5$[D-Tyr(Et)^2, Trp^3]VAVP	7.61	34
5	$d(CH_2)_5$[D-Tyr(Et)^2, Hphe^3]VAVP	7.59	85
6	$d(CH_2)_5$[D-Tyr(Et)^2, Tic^3, Tyr-NH_2^9]VAVP	7.57	16.6
7	$d(CH_2)_5$[D-Tyr(Et)^2, Tic^3, D-Cys^6]VAVP	7.52	14.5
8	$d(CH_2)_5$[D-Tyr(Et)^2, Pro^3, Tyr-NH_2^9]VAVP		2.01

[a]Binding affinity data for hV_2R are presented for the first time in this review.

[b]Estimated in vivo pA_2 values represent the negative logarithm of the ED divided by the estimated volume of distribution (67 ml/kg).

[c]The anti-V_2 (rat) data are from Manning et al., 1997 and references therein.

V_2 Antagonist with high affinity for human V_2 receptor

The development of peptide AVP V_2/V_{1a} antagonists for use as aquaretics in humans was abandoned at Smith Kline Beecham following the discovery that the lead V_2/V_{1a} candidate, desGly, $d(CH_2)_5$[D-Tyr(Et)^2,Val^4]AVP (Manning et al., 1984) (peptide 2, Table 13) was an agonist in humans (Ruffolo et al., 1991) (Table 14). With the emergence of non-peptide V_2 antagonists (see below), no further attempts were made to find a peptide V_2 antagonist which would be an effective V_2 antagonist in humans. We now report the discovery of a new potential candidate, $d(CH_2)_5$[D-Tyr(Et)^2, Pro^3]VAVP (peptide 2, Table 14). We previously reported that this peptide is one of a series of a potent V_2/V_{1a} antagonists in the rat (Manning et al., 1997). We now report that this peptide exhibits a very high affinity ($K_i = 0.5$ nM) for the human V_2 receptor. This peptide could be a promising lead to the design of peptide V_2 antagonists which are effective in humans.

Development of highly selective OT antagonists in the rat and in humans

Since this subject was last reviewed (Manning and Sawyer, 1993), we have designed and synthesized a number of highly selective OT antagonists (Manning et al., 1995a; Chan et al., 1996; Manning et al., 2001, 2005). A number of these are slowly being adopted as substitutes for one or our original OT antagonists, d$(CH_2)_5$[Tyr(Me)^2]OVT (peptide 2, Table 15). In the rat, this OTA is actually 5 times more potent as a V_{1a} antagonist than as an OT antagonist (Bankowski et al., 1980). Thus this OT antagonist is not suitable for discriminating between OT and V_{1a} receptors in the rat. Unfortunately, It has been widely misused as a "selective" OT antagonist. The OT antagonist No. 4, d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Tyr–NH_2]OVT (OTA)

Table 15. Some non-selective, selective and highly selective oxytocin antagonists in the rat

No.	Peptide	Antioxytocic (anti-OT)			Antivasopressor (anti-V_{1a})		Antidiuretic activity (V_2) (U/mg)	ED ratio[d]
		In vitro pA_2[a]	In vivo		ED[b]	pA_2[c]		
		No Mg^{2+}	ED[b]	pA_2[c]				
1	Atosiban, d[D-Tyr(Et)^2, Thr^4]OVT[e,f]	8.29[k] 7.71[h]	Antagonist[g] 5.95[h]	7.05[h]	Agonist[k] 0.02 (i.u./μmol) 48.5[h]	6.14[h]	Agonist[k] 0.04 (i.u./μmol) antagonist (p$A_2\approx 5.9$)[h]	8
2	d$(CH_2)_5$[Tyr(Me)^2]OVT[i]	8.52	4.2	7.37	0.80	7.96	≈0.01	0.2
3	desGly–NH_2,d$(CH_2)_5$ [Tyr(Me)^2, Thr^4]OVT[k]	7.89	1.3	7.69	23	6.48	Antagonist (p$A_2\approx 5.5$)	17.7
4	d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Tyr–NH_2^9]OVT[j]	7.63	1.0	7.83	6.6	7.02	≈0.015	6.6
5	desGly–NH_2,d$(CH_2)_5$ [D-Tyr^2, Thr^4]OVT[h]	7.77	2.85	7.37	272	5.39	Antagonist (p$A_2<5.5$)	95
6	desGly–NH_2,d$(CH_2)_5$ [D-Trp^2, Thr^4]OVT[h]	8.02	3.08	7.34	248	5.43	≈0.01	80
7	desGly–NH_2,d$(CH_2)_5$ [D-Trp^2, Thr^4, Dap^5]OVT[l]	7.47			n.d.	<0.005	Infinite	
8	d$(CH_2)_5$[D-Thi^2, Thr^4, Tyr–NH_2^9]OVT[m]	7.72	4.55	7.19	≈700	≈5	≈0.02	≈147
9	desGly–NH_2,d$(CH_2)_5$ [D-2-Nal^2, Thr^4]OVT[n]	7.66	8.73	6.89	17.9	6.59	pA_2 6.99	2.1
10	desGly–NH_2,d$(CH_2)_5$[D-2-Nal^2, Thr^4, Tyr–NH_2^9]OVT[n]	7.73	5.37	7.10	1.36	6.70	pA_2 7.17	0.3

[a]In vitro pA_2 values represents the negative logarithm to the base 10 of the average molar concentration [M] of antagonist which reduces the response to 2x units of agonist to the response with 1x units of agonist.
[b]The ED is defined as the dose (in nmol/kg) of antagonist that reduces the response to 2x units of agonist to the response with 1x units of agonist administered in the absence of antagonist.
[c]Estimated in vivo pA_2 values represent the negative logarithms of the ED divided by the estimated volume of distribution (67 ml/kg).
[d]ED ratio = antivasopressor ED/antioxytocic ED.
[e]Atosiban is the trade name for [1-deamino, 2-*O*-ethyl-D-tyrosine, 4-threonine]-ornithine-vasotocin.
[f]Data from Melin et al., 1986.
[g]In vivo antagonistic potency was expressed as a percentage of the standard. ED values were not reported in Melin et al., 1986.
[h]Data from Manning et al., 1995a.
[i]Data from Bankowski et al., 1980.
[j]Data from Elands et al., 1988.
[k]Original synthesis and pharmacological data reported in Manning et al., 1989.
[l]Chan et al., 1996.
[m]Data from Manning et al., 2001.
[n]Data from Manning et al., 2005.

(Manning et al., 1989) has also found widespread use as a "selective" OT antagonist. However, as the data in Table 15 clearly shows, with an ED ratio of 6.6 it is clearly not highly selective. The [^{125}I]OTA is widely used as a probe for OT receptors (see Table 17) (Elands et al., 1988). Peptide 3, desGly–NH$_2$, d(CH$_2$)$_5$[Tyr(Me)^2, Thr^4]OVT, is far more selective for OT receptors than for V$_{1a}$ receptors when compared to d(CH$_2$)$_5$[Tyr(Me)^2]OVT. In fact, it is about 18 times more potent as an OT antagonist in the rat than as a V$_{1a}$ antagonist (Manning et al., 1989). It has been used very effectively in a wide variety of studies by Inga Neumann and her colleagues (Neumann et al., 2003, 2006). desGly–NH$_2$, d(CH$_2$)$_5$[D-Tyr^2, Thr^4]OVT (peptide 5) is one of the most selective OT antagonists reported to date (Manning et al., 1995a). It is 95 times more potent as an OT antagonist than as a V$_{1a}$ antagonist in the rat. This selective OT antagonist has been utilized in a variety of studies (Pedersen and Boccia, 2002; Detillion et al., 2004; Smith et al., 2006; Hagedorn et al., 2007; McKee et al., 2007). Although somewhat less potent in vivo than all of the other OTAs, other than Atosiban (peptide 1), peptide 8, d(CH$_2$)$_5$,[D-Thi^2,Thr^4,Tyr–NH$_2^9$]OVT (Manning et al., 2001), because it is such a weak V$_{1a}$ antagonist, is one of the most selective OT antagonists reported to date. It is almost 150 times more potent as an OT antagonist than as a V$_{1a}$ antagonist. Although this OT antagonist has been supplied to a number of investigators, to date no reports on the use of this peptide have been published. Peptide 7, desGly–NH$_2$, d(CH$_2$)$_5$[D-Trp^2, Thr^4, Dap^5]OVT is a potent OT antagonist in vitro (Chan et al., 1996). It exhibits undetectable V$_{1a}$ antagonism. Thus in vitro, it exhibits infinite selectivity for OT versus V$_{1a}$ receptors. Based on studies reported to date, peptides 3, desGly–NH$_2$,d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4]OVT and 5, desGly–NH$_2$,d(CH$_2$)$_5$[D-Tyr^2,Thr^4]OVT offer clear advantages over d(CH$_2$)$_5$[Tyr(Me)^2]OVT (peptide 2) and Atosiban for in vivo studies requiring the discrimination of OT versus V$_{1a}$ receptors in the rat.

OT Antagonists with high affinities and selectivities for the human OT receptor

Table 16 contains a number of new OT antagonists which have strikingly higher affinities for the human receptor than the peptide OT antagonist, Atosiban (Manning et al., 2005). To date, Atosiban is the only OT antagonist, peptide or non-peptide which has been approved (in Europe) for clinical use as a tocolytic or the prevention of

Table 16. Affinity constants for human OT and VP V$_{1a}$ receptors of D-2-Nal^2 and 2-Nal^2 OT-antagonists (1–4), d(CH$_2$)$_5$[Tyr(Me)^2] OVT (A), OTA (B) and atosiban

No.	Peptide	hOTR K_i (nM)[c]	hV$_{1a}$R K_i (nM)[c]	Receptor selectivity hV$_{1a}$R/hOTR	hOT receptor affinity versus atosiban	hReceptor selectivity versus atosiban
	Atosiban[a,b]	76.4	5.1	0.07	1	1
A	d(CH$_2$)$_5$[Tyr(Me)^2]OVT[d,e]	3.49	32.7	9.4	0.045	134
1	desGly–NH$_2$,d(CH$_2$)$_5$[D-2-Nal^2, Thr^4]OVT[f]	0.17	1.1	6.5	0.002	93
2	desGly–NH$_2$,d(CH$_2$)$_5$[2-Nal^2, Thr^4]OVT[f]	0.29	1.3	4.5	0.004	64
B	d(CH$_2$)$_5$[Tyr(Me)^2, Thr^4, Tyr–NH$_2^9$]OVT[f]	0.59	5.26	8.9	0.008	127
3	d(CH$_2$)$_5$[D-2-Nal^2, Thr^4, Tyr–NH$_2^9$]OVT[f]	0.07	0.19	2.7	0.0009	39
4	d(CH$_2$)$_5$[2-Nal^2, Thr^4, Tyr–NH$_2^9$]OVT[f]	0.14	0.54	3.9	0.002	56

[a]Original synthesis reported in Melin et al., 1986.
[b]Data reported here was obtained with atosiban re-synthesized in the Manning laboratory (Manning et al., 1995a).
[c]K_i, concentration of peptide leading to half-maximal specific binding deduced from competition experiments.
[d]Original synthesis reported in Bankowski et al., 1980.
[e]Barberis and Manning (unpublished data).
[f]Data from Manning et al., 2005.

premature labour (Romero et al., 2000; Valenzuela et al., 2000; Vatish and Thornton, 2002; Tsatsaris et al., 2004).

Peptides 1–4 (Table 16), the four most promising new OT antagonists, exhibit gains in human OT receptor affinity relative to Atosiban of 449, 263, 1091 and 546. These four peptides are also more selective for the human OT receptor than for the human V_{1a} (vascular) receptor relative to Atosiban and exhibit selectivity gains of 93, 64, 39 and 56 respectively. These new OT antagonists clearly possess a superior and safer pharmacological profile as potential tocolytic agents than Atosiban. They are thus promising new candidates for development as potential tocolytic agents for the prevention of premature labour. In this regard, it should be noted that Ferring has recently reported a new peptide OT antagonist, FE200440 (Barusiban) (Nilsson et al., 2003) which also exhibits much higher affinities and selectivities for the hOTR than Atosiban. Barbusiban is currently being evaluated as a potential tocolytic in non-human primates (Reinheimer et al., 2005; Reinheimer, 2007).

In Table 16, we present human OT and V_{1a} receptor affinity data (C. Barberis; personal communication) for the widely used OT antagonist $d(CH_2)_5[Tyr(Me)^2]OVT$ (Bankowski et al., 1980). With a hOTR $K_i = 3.49$ nM, this peptide exhibits a gain in affinity of 22 relative to Atosiban. With a $hV_{1a}R$ $K_i = 32.7$ nM, its affinity for the $hV_{1a}R$ is lower than that of Atosiban. It thus exhibits a gain in $OTR/V_{1a}R$ selectivity of 134 relative to Atosiban. The rat and human data for $d(CH_2)_5[Tyr(Me)^2]OVT$ in Tables 15 and 16 are more examples of striking species differences. Thus, in the rat this peptide is five times more potent as a V_{1a} antagonist, than as an OT antagonist: whereas in humans, its affinity for the OT receptor is over nine times higher than for the V_{1a} receptor.

Radiolabelled ligands for rat and/or human vasopressin and oxytocin receptors

Tritiated and radioiodinated ligands for AVP and OT receptors (Table 17) have found widespread use in receptor localization and identification studies (for references see Manning et al., 1995b). Until the discovery of radioiodinated ligands, these studies relied solely on tritiated ligands. While tritiated OT is specific for rat and human OT receptors, tritiated AVP and LVP lack specificity for a single receptor subtype. The tritiation of the selective OT receptor agonist, $[Thr^4,Gly^7]OT$ (Elands et al., 1988), the selective V_2 receptor agonist dDAVP (Marchingo et al., 1988), the selective V_{1a} antagonist, $d(CH_2)_5[Tyr(Me)^2]AVP$ (Howl et al., 1991) and the selective V_2 antagonist, desGly–NH_2, $d(CH_2)_5[\text{D-Ile}^2, Ile^4]AVP$ (Trinder et al., 1991) led to labelled ligands which are highly selective for rat OT, V_{1a} and V_2 receptors (Table 17). Radioiodinated ligands offer significant advantages over tritiated peptides. They are easier and less expensive to prepare. Also, they possess much higher specific radioactivities which can improve the sensitivity and accuracy of receptor binding studies. Furthermore, radioiodination permits much more rapid and definitive radioautograhic localization of receptors. Table 17 lists a number of radioiodinated ligands for rat and/or human OT, V_{1a} and V_2 receptors which we and others have developed. Two in particular, $[^{125}I]$OTA (Elands et al., 1988) (No. 9) and $[^{125}I]$HO–LVA (Barberis et al., 1995) (No. 14) have found widespread use as selective probes respectively of OT and V_{1a} rat and human receptors. Peptide 10 $[^{125}I]$ $d(CH_2)_5[\text{D-Tyr(Et)}^2, Val^4, Tyr\text{–}NH_2^9]AVP$ is a high affinity non-selective radioiodinated ligand for the human V_2 receptor (Ala et al., 1997). Peptide 11, $[^{125}I]$ $d(CH_2)_5[\text{D-Ile}^2,Ile^4,Tyr\text{–}NH_2^9]AVP$ (Manning et al., 1995b; Barberis et al., 1999) is a high affinity somewhat selective ligand for the rat V_2 receptor. It has been utilized in studies by Cotte et al. (1998) and Tian et al. (2000). Preliminary affinity data for the rat and human V_2 receptors for a variety of peptide and $[^{125}I]$ peptide V_2 antagonists are reported in (Manning et al., 1995b). With the exception of the aforementioned $[^{125}I]$ $d(CH_2)_5[\text{D-Tyr(Et)}^2, Val^4, Tyr\text{–}NH_2^9]AVP$ (Ala et al., 1997), all of these peptides reveal striking species differences between the rat and human V_2 receptors. There is thus still a need for a high affinity selective radioiodinated ligand for the rat and human V_2 and V_{1b} receptors. Sanofi has reported

Table 17. Radiolabelled ligands for rat and/or human vasopressin and oxytocin receptors

No.	Radiolabelled ligand	Dissociation constants K_d (nM) for rat VP/OT receptors			
		Rat kidney (rV_2R)	Rat liver ($rV_{1a}R$)	Rat hypophysis ($rV_{1b}R$)	Rat uterus (rOTR)
1	[^{3}H]-AVP[a]	0.4	0.6–3	1–3	1.7
2	[^{3}H]-LVP[a]	7	8	4	1.7
3	[^{3}H]-OT[a]	370	78	250	1.0–2.5
4	[^{3}H]-[Thr^4, Gly^7]OT[a]	> 10,000	> 8000		1
5	[^{3}H]desGly^9 ,d(CH$_2$)$_5$[D-Tyr(Et)^2]VAVP[a]	0.4	0.2		
6	[^{3}H]desGly–NH$_2^9$,d(CH$_2$)$_5$[D-Ile^2, Ile^4]AVP[b]	2.8			
7	[^{3}H]d(CH$_2$)$_5$[Tyr(Me)^2]VAVP[a]		0.3		
8	[^{3}H]-dDAVP[a]	0.8			
9	[^{125}I]d(CH$_2$)$_5$[Tyr(Me)^2, Thr^4, Tyr–NH$_2^9$]OVT ([^{125}I-OTA)[a]	10.2	13.6		0.03
10	[^{125}I]d(CH$_2$)$_5$[D-Tyr(Et)^2, Val^4, Tyr–NH$_2^9$]AVP[c]	1.12	0.33	> 1000	11.0
11	[^{125}I]d(CH$_2$)$_5$[D-Ile^2, Ile^4, Tyr–NH$_2^9$]AVP[d,e,j]	0.32			
12	[^{125}I]Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Arg–Tyr–NH$_2$[a]	62	0.18	92	1.4
13	[^{125}I]Phaa–D-Tyr(Et)–Phe–Gln–Asn–Lys–Pro–Arg–Tyr–NH$_2$[f,g]		0.168		
14	4-HO–Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Arg–NH$_2$ (HO–LVA)[f]	137	0.078	5.9	2.7
15	[^{125}]HO–LVA[h]	35.5	0.008	4.5	1.9
16	Phaa–D-Tyr(Et)–Phe–Val–Asn–Lys–Pro–Tyr–NH$_2$[i]		0.52		

[a]Data from Schmidt et al., 1991.
[b]Data from Trinder et al., 1991.
[c]Data (human) from Ala et al., 1997.
[d]Data from Cotte et al., 1998.
[e]Human V_2R $K_i = 128$.
[f]For original synthesis see Manning et al., 1991.
[g]Data from Strakova et al., 1997.
[h]Data from Barberis et al., 1995.
[i]Data (human) from Thibonnier et al., 1993.
[j]Human V_{1a}, V_{1b}, V_2 and OT receptor data reported in Barberis et al., 1999.

the synthesis and properties of the tritiated non-peptide V_2 antagonist Satavaptan (SR-121463) (Serradeil-Le Gal et al., 2000) and the tritiated non-peptide V_{1b} antagonist SR-149415 (Serradeil-Le Gal et al., 2007). These tritiated ligands promise to be useful tools for studies on the rat and human V_2 and V_{1b} receptors. However, high non-specific binding, due to the hydrophobicity of the tritiated SR-149415, will preclude its usefulness for the detection of low levels of V_{1b} receptors by autoradiographic techniques.

High affinity fluorescent agonists and antagonists for human OT and AVP receptors

Previous studies have shown the value of fluorescent AVP analogues for studies on rat and human AVP receptors (Buku et al., 1988, 1989; Eggena and Buku, 1989; Buku and Gazis, 1990; Lutz et al., 1990; Guillon et al., 1992; Howl et al., 1993; Thibonnier et al., 1993). Tables 18 and 19 list some new fluorescent agonists and antagonists for the human OT and AVP receptors. The fluorescent OT agonists in Table 18 exhibit very high affinities and selectivities for the human OT receptor (Terrillon et al., 2002). These fluorescent OT agonists are very useful tools for studies on desensitization and the possible internalization of the ligand OT receptor. These fluorescent ligands can also be used to perform fluorescence recovery after photobleaching to study the diffusion of molecules in a tissue or in one cell and to obtain structural information for ligand receptor interactions for fluorescence quenching, polarization or resonance energy transfer experiments (Terrillon et al., 2002). The novel fluorescent linear antagonists in Table 19 exhibit high affinities and

selectivity for the human AVP V_{1a} receptor (Durroux et al., 1999). These linear fluorescent V_{1a} receptor ligands will be useful tools for studies on the structural basis of ligand recognition by AVP receptors. These two high affinity human V_{1a} receptor ligands can be used in cellular and subcellular analysis and co-localization with other markers in fluorescence microscopy. The fluorescent linear V_{1a} human receptor antagonist No. ♯1 (Table 19) can be readily used for flow cytoflowmetric cell analysis and cell sorting. It may thus offer advantages over iodinatable ligands for screening new molecules acting on human AVP receptors. One of these fluorescent linear V_{1a} antagonists (Rhm[8]PVA) No. 2 (Table 19) has been utilized to visualize AVP V_{1a} receptors in the rat liver (Tran et al., 1999).

Table 18. High-affinity fluorescent agonists for human AVP and OT receptors[a]

No.	Peptide	Affinity K_i (nM)			
		OTR	V_{1a}R	V_{1b}R	V_2R
	OT	0.8	120	n.d.	3500
1	d[Lys[8](5/6C-Flu)]VT	0.29	57	124	> 10,000
2	d[Orn[8](5/6C-Flu)]VT	0.25	107	393	> 10,000
3	[HO[1]][Lys[8](5/6C-Flu)]VT	0.34	13.7	66	n.d.
4	[HO[1]][Orn[8](5/6C-Flu)]VT	0.18	12.2	126	n.d.
5	[HO[1]][Orn[8](5/6C-Rhm)]VT	0.09	7.6	n.d.	n.d.

Note: n.d., not determined. 5/6C-Flu, 5 (or 6)-carboxyfluoresceinyl; 5/6C-Rhm, 5-(or 6)-carboxytetramethylrhodamyl.
[a]Data reported in Terrillon et al., 2002.

Bivalent ligands for human and rat OT and V_{1a} receptors

As part of a study on chimeric peptides, Wheatley and colleagues reported the first bivalent ligands of a linear V_2/V_{1a} antagonist for the rat V_{1a} and V_2 receptors (Howl et al., 1997) (Table 20). We recently reported the syntheses and some preliminary pharmacological properties of bivalent ligands for the hOTR, V_{1a} and V_{1b} receptors (Chini et al., 2007; Chini and Manning, 2007). Suberic acid, (HOOC–$(CH_2)_6$–COOH) utilized as reported by Gera et al. (1996), served as the spacer joining Orn or Lys residues in an OT/V_{1a} antagonist d$(CH_2)_5$[Tyr(Me)[2]]OVT, and two linear V_{1a}/OT antagonists, HO–Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Lys–NH_2, and HOPhaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Arg–Lys–NH_2. The resulting suberyl-(OC–$CH_2)_6$–CO–) bivalent ligands exhibit high affinities, in the nanomolar range, for the hOTR and V_{1a} receptors expressed in heterologous cell systems. As OTR/V_{1a}, OTR/V_{1b} and V_{1a}/V_{1b} heterodimers may be postulated to exist in the central nervous system as well as in peripheral organs and tissues (Terrillon et al., 2003), the pharmacological potential of bivalent ligands now needs to be explored. However, the lack of experimental evidence for the formation, in vivo, of dimers or heterodimers and the difficulty of setting-up pharmacological assays for the screening and identification of the properties of bivalent ligands, represent real challenges in this field. In fact, as in the case of bivalent ligands targeting opioid receptors (Daniels et al., 2005;

Table 19. High affinity fluorescent linear antagonists for human OT and AVP receptors[a]

No.	Peptide (abbreviation)	Receptor subtype			
		V_{1a}	V_{1b}	V_2	OT
	AVP	1.7	1.1	1.1	1.65
1	4-HO–Ph$(CH_2)_2$-D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Lys(5C-Flu)–NH_2 ([Lys[8](5C-Flu)]PVA[b]	0.17	142	9,200	1.6
2	4-HO–Ph$(CH_2)_2$-D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Lys(5C-Rhm)–NH_2 ([Lys[8](5C-Rhm)]PVA)[b]	0.07	27	7,500	1.2

Note: CHO, Chinese hamster ovary cells; [^{125}I]HO–LVA, [^{125}I] 4-HO-Phaa-D-Tyr(Me)–Phe-Gln–Asn–Arg–Pro–Arg–NH_2; [^{125}I]OTA, [^{125}I]oxytocin antagonist, d$(CH_2)_5$[Tyr(Me)[2], Thr[4], Tyr-NH_2^9]OVT; 5C-Flu, 5-carboxyfluoresceinyl; 5C-Rhm, 5-carboxytetramethylrhodamyl.
[a]Data reported in Durroux et al., 1999.
[b]The inhibition constants (K_i in nM) of peptides No.1 and No.2 were determined by displacement experiments using membranes of CHO cells expressing one subtype of human receptor and the convenient radiolabelled ligand: [^{125}I]HO–LVA for V_{1a} and V_{1b} receptors, [^{125}I]OTA for oxytocin receptor, and [$^3H_3$]AVP for V_2 receptor.

Table 20. Human OTR, V_{1a}R and V_{1b}-R affinities of the dimeric cyclic and linear OT/VP antagonists (numbers 1–3) compared with those of atosiban

No.	Peptide	Affinity (K_i)[a,b] (nM)				
		hOTR	$hV_{1b}R$	$hV_{1a}R$	$rV_{1a}R$	bV_2R
	Atosiban[c] (d[D-Tyr(Et)^2, Thr^4]OVT)	76.4[c]		5.1[c]		
A	CO^8[Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Lys–Tyr–NH_2]$_2$[b]				0.29	330
B	Sub^8[Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Lys–Tyr–NH_2]$_2$[b]				6.3	>10
1	Sub^8[d(CH_2)$_5$ [Tyr(Me)^2, Orn^8]VT]$_2$[a]	26.8	>1000	52		
2	Sub^8[HO–Phaa-D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Lys–NH_2]$_2$[a]	4.03	3.6	5.15		
3	Sub^9[HO–Phaa–D-Tyr(Me)–Phe–Gln–Asn–Arg–Pro–Arg–Lys–NH_2]$_2$[a]	4.63	n.d.[d]	0.40		

Sub^{8,9}, suberyl linker (OC–(CH_2)$_6$CO–) joining Orn or Lys residues at positions 8 or 9.
[a]Competition binding experiments for human (h) receptors were performed on cell homogenates of transiently transfected COS7 cells as described in Chini et al., 2007. K_i values for the hOTR were obtained by means of displacement of [^{3}H]OT. K_i values for the $hV_{1a}R$ and $hV_{1b}R$ were obtained by means of displacement of [^{3}H]AVP.
[b]The binding affinity of peptides A and B were determined on rat (r) liver membranes and bovine (b) kidney medulla membranes as reported in Howl et al., 1997 and references cited therein. K_i values for the $rV_{1a}R$ and bV_2R were obtained by means of displacement of [Phe-3,4,5-^{3}H]AVP.
[c]Data from Manning et al., 2005.
[d]n.d., not determined.

Peng and Neumeyer, 2007), bivalent ligands may be characterized by novel features that may lead to the generation of a new class of therapeutics.

Non-peptide vasopressin and oxytocin antagonists and agonists

Since the discovery of the first non-peptide V_{1a} antagonist by Otsuka (Yamamura et al., 1991), at least 10 other pharmaceutical companies have published non-peptide V_{1a}, V_{1b}, V_2, V_2/V_{1a} and OT antagonists. Also, more recently, at least three companies have reported non-peptide agonists for the V_2 receptor and for the OT receptor. To date, there are no reports on non-peptide V_{1a} or V_{1b} agonists. The current status of the development and clinical trials on non-peptide antagonists and agonists is summarized below and in Tables 21–24. As noted above, the non-peptide OT antagonists and non-peptide AVP antagonists are reviewed respectively in (Freidinger and Pettibone, 1997) and in (Serradeil-Le Gal et al., 2002b). The original references cited in these two reviews and those cited here can be consulted for more details on the structures, syntheses, pharmacological properties, species differences, lack of receptor selectivity, pre-clinical studies and when carried out, clinical trials for the non-peptide AVP and OT antagonists and agonists listed in Tables 21–24.

Non-peptide V_{1a} receptor antagonists

Following the report in 1991 by the Otsuka company of the first non-peptide V_{1a} antagonist OPC-21268 (Yamamura et al., 1991) a number of other companies, most notably Sanofi-Synthelabo, Yamanouchi, and Fujisawa (the two latter now combined as Astellas) have developed other non-peptide V_{1a} antagonists (Table 21). Among the most promising are SR49059 (Relcovaptan), developed by Sanofi in 1993 (Serradeil-Le Gal et al., 1993), YM218, developed by Yamanouchi in 2005 (Tahara et al., 2005), and SRX251, developed in 2006 by Azevan (Ferris et al., 2006; Guillon et al., 2007a, b). As an example of both species differences and lack of receptor selectivity, the Otsuka V_{1a} antagonist OPC-21268 was subsequently shown to have a poor affinity for the human V_{1a} receptor (Serradeil-Le Gal et al., 1993) and to also have affinity for the rat and human OT receptors (Freidinger and Pettibone, 1997). Thus, although OPC-21268 was ineffective in humans, it served as an excellent lead to the design, at Merck, of promising non-peptide OT antagonists (Freidinger and Pettibone, 1997) and to the design at Otsuka of

Table 21. Non-peptide vasopressin antagonists

No.	Company	Code	Name	Status
Receptor type: V_{1a}				
1	Otsuka	OPC-21268[a]	None	Phase II Japan stopped US/Europe
2	Sanofi-Synthelabo	SR49059[b]	Relcovaptan	Phase II (stopped)
3	Azevan	SRX251[c]	None	Phase I
4	Astellas	YM-218[d]	None	Pre-clinical
Receptor type: V_{1b}				
1	Sanofi	SSR149415[e]	None	Pre-clinical
2	Organon	ORG 52186[p]	None	Pre-clinical
Receptor type: V_2				
1	Otsuka	OPC-31260[f]	Mozavaptan	Phase II
2	Otsuka	OPC-41061[g]	Tolvaptan	Phase III
3	Sanofi	SR121463(B)[h]	Satavaptan	Phase III
4	Wyeth-Ayerst	VPA-985[i]	Lixivaptan	Phase III
5	Wyeth-Ayerst	WAY-140288[j]	None	Pre-clinical
6	Wakamoto	VP-343 and VP-339[k]	None	Pre-clinical
7	Wakamoto	VP-365[k]	None	Pre-clinical
8	Astellas	FR-161282[k]	None	Pre-clinical
9	Johnson and Johnson	RWJ-351647[l]	None	Pre-clinical
Receptor type: V_2/V_{1a}				
1	Astellas	YM-087[m]	Conivaptan	Approved by US FDA i.v. use
2	Astellas	YM-471[n]	None	Pre-clinical
3	Wyeth-Ayerst	CL-385004[o]	None	Pre-clinical

Note: For original synthesis, structure and pharmacological properties see the following references:
[a]Yamamura et al., 1991.
[b]Serradeil-Le Gal et al., 1993.
[c]Ferris et al., 2006; Guillon et al., 2007a.
[d]Tahara et al., 2005.
[e]Serradeil-Le Gal et al., 2002a, b, 2005.
[f]Yamamura et al., 1992.
[g]Yamamura et al., 1998.
[h]Serradeil-Le Gal et al., 1996.
[i]Albright et al., 1998.
[j]Ashwell et al., 2000.
[k]Serradeil-Le Gal et al., 2002b.
[l]Gunnet et al., 2006.
[m]Tahara et al., 1997.
[n]Tsukada et al., 2002.
[o]Aranapakam et al., 1999.
[p]Letourneau et al., 2006; Craighead and MacSweeney, 2008.

the first non-peptide V_2 antagonist OPC-31260 (Mozavaptan) (Yamamura et al., 1992). AVP V_{1a} antagonists have potential as therapeutic agents for the treatment of Raynaud's disease, hypertension, CHF, brain oedema, motion sickness, oncology, (small cell lung carcinoma), dysmenorrhea, preterm labour, anger reduction (serenics). The Sanofi V_{1a} antagonist SR49059 (Relcovaptan) has a high affinity for both rat and human V_{1a} receptors. While it has proved to be very valuable in proof of concept studies on the role of AVP in a number of the above diseases, its clinical development has been discontinued (Serradeil-Le Gal et al., 2002b). Given the early promise of non-peptide V_{1a} antagonists as therapeutic agents, it is somewhat surprising that to date, with the exception of SRX251 (Ferris et al., 2006; Guillon et al., 2007a), there are no clinical trials underway with any of the many non-peptide V_{1a} antagonists which have emerged from pre-clinical studies. The

development of SRX251 for the treatment of aggression and anger management is potentially very exciting (Ferris et al., 2006).

Non-peptide V_2 antagonists (Vaptans, Table 21)

The report by Otsuka in 1992 of the first non-peptide V_2 antagonist OPC-31260 (Mozavaptan) (Yamamura et al., 1992), elicited much interest in the development of other non-peptide V_2 antagonists for the treatment of hyponatremia at six other pharmaceutical companies. To date, these studies have uncovered a variety of orally active non-peptide V_2 antagonists shown to be effective as aquaretics in animals and in humans. As a class, these have been termed Vaptans. (For reviews see: Thibonnier et al., 2001; Serradeil Le Gal, 2002b; Orlandi et al., 2005; Palm et al., 2006; Streefkerk and van Zwieten, 2006; Verbalis, 2006; Cawley, 2007; Gines, 2007; Chen et al., 2007; Parashar et al., 2007; Shackel and McCaughan, 2007.) The following are some of the most promising Vaptans uncovered to date.

1. OPC-41061 (Tolvaptan) developed by Otsuka (Yamamura et al. (1998).
2. SR-121463 (Satavaptan) developed by Sanofi (Serradeil-Le Gal et al., 1996).
3. VPA-985 (Lixivaptan), developed by Wyeth-Ayerst (Albright et al., 1998). All three Vaptans are currently in phase III clinical trials. Although none to date has received US FDA approval. The clinical trials with Tolvaptan (Schrier et al., 2006; Gheorghiade et al., 2007); Satavaptan (Soupart et al., 2006) and Lixivaptan (Abraham et al., 2006) have shown promise for the treatment of hyponatremia, liver cirrhosis, CHF and polycystic kidney disease. Consequently, the encouraging results reported to date have elicited many reviews and reports on their efficacy in a wide variety of journals (see for example: Orlandi et al., 2005; Palm et al., 2006; Streefkerk and van Zwieten, 2006; Verbalis, 2006; Ali et al., 2007; Cawley, 2007; Gines, 2007; Munger, 2007; Parashar et al., 2007; Shackel and McCaughan, 2007).

   A number of other non-peptide V_2 antagonists are currently in pre-clinical development. These include:
4. Way-140288 (Wyeth-Ayerst) (Ashwell et al., 2000).
5. VP-343, VP-339 both (Wakamoto) (Serradeil Le-Gal, 2002b)
6. FR-161282, (Fujisawa) (Serradeil-Le Gal, 2002b)
7. RWJ-351647 (Johnson and Johnson) (Gunnet et al., 2006).

Non-peptide V_2/V_{1a} antagonists

The dual non-peptide V_2/V_{1a} antagonist YM-087 (Conivaptan), developed at Yamanouchi (Tahara et al., 1997, 1998) and marketed under the name Vaprisol by Astellas Pharma is the only Vaptan approved by the US FDA for i.v. use in the treatment of hyponatremia (Verbalis, 2006; Walter, 2007) (Table 21). This dual non-peptide V_2/V_{1a} antagonist thus holds the distinction of being the first non-peptide AVP antagonist approved for clinical use. It is somewhat ironic, that in view of all the advantages of its oral bioavailability, it was approved for i.v. administration and not for oral administration. Conivaptan is also used in clinical trial for the treatment of heart failure (Schwarz and Sanghi, 2006).

In December 2006 the Veterans Health Administration's Pharmacy Benefits Management (PBM) Strategic Health Care Group published a National PBM drug monograph entitled "Conivaptan hydrochloride injection (Vaprisol®)". This monograph, which can be accessed at www.pbm.va.gov addresses the benefits and risks involved in the use of Conivaptan to treat euvolemic hyponatremia and CHF by i.v. administration in a hospital setting. In noting that Conivaptan is a potent cytochrome CYP3A4 inhibitor and substrate, caution is urged in the administration of this drug with other drugs which inhibit this enzyme and points to this concern as the main reason that Conivaptan was approved for i.v. rather than oral administration. Finally, following a thorough analysis of the clinical data, this monograph does not recommend that patients with envolemic

hyponatremia be treated with Conivaptan "until it has been found to be superior to time proven safe and effective therapies such as free water restriction and/or intravenous saline". Furthermore, until safety issues are addressed, the monograph concludes that Conivaptan should not become "standard therapy' for the treatment of CHF. So clearly, further studies are need to prove the long-term safety and efficacy of Conivaptan. Thus its approval by oral administration is still very much in doubt. Other non-peptide V_2/V_{1a} antagonists, currently in preclinical development, include YM-471 (Astellas) (Tsukada et al., 2002) and CL-385004 (Wyeth Ayerst) (Aranapakam et al., 1999).

Non-peptide V_{1b} antagonists

To date, only two companies, Sanofi, and Organon have reported success in this area (Table 21). The first non-peptide V_{1b} antagonist SSR-149415 was reported in 2002 (Serradeil-Le Gal et al., 2002a, b). SSR-149415 is orally active and was reported to possess high and selective affinity for rat and human V_{1b} receptors in vitro and in vivo (Serradeil-Le Gal et al., 2002a, b, 2005). This non-peptide V_{1b} antagonist was subsequently shown to also exhibit human receptor OT antagonism (Griffante et al., 2005). It is currently in pre-clinical development for the treatment of anxiety and depression. It has been shown to decrease anxiety in rats and exerts marked antidepressant-like activity in several predictive animal models (Griebel et al., 2002). More recently, Organon has obtained a patent for a highly promising orally active V_{1b} antagonist, ORG-52186 (Letourneau et al., 2006). This was very recently reported to be effective in the reduction of chronic stress and aggression in the rat (Craighead and MacSweeney, 2008). ORG-52186 possesses a high affinity and selectivity for the human V_{1b} receptor. It also possesses a high affinity for the rat V_{1b} receptor and is a V_{1b} antagonist in both species (personal communication from Mark Craighead). The non-peptides SSR-149415 and ORG-52186 are valuable new tools for studying animal and human V_{1b} receptors. In this regard, they very much compliment the selective V_{1b} peptide agonist ligands noted above — namely d[Cha^4]AVP, selective for the human V_{1b} receptor (Derick et al., 2002; Guillon et al., 2004) and d[Leu^4,Lys^8]VP, selective for the rat V_{1b} receptor (Pena et al., 2007a, b).

Non-peptide oxytocin antagonists

The emergence of orally active non-peptide OT antagonists at the Merck laboratories in the early nineties (Evans et al., 1992) appeared to fill a therapeutic void for an effective orally active tocolytic for the treatment and prevention of premature labour (Table 22). However, due to

Table 22. Non-peptide oxytocin antagonists

No.	Company	Code	Name	Status
1	Merck	L-368,899[a]	None	Phase II discontinued
2	Merck	L-371,257[b]	None	Discontinued
3	Merck	L-372,662[c]	None	Phase II discontinued
4	Wyeth-Ayerst	WAY-162720[d]	None	Pre-clinical
5	Serono	None[e]	None	Pre-clinical
6	Sanofi	SSR126768A[f]	None	Pre-clinical
7	GlaxoSmithKline	GSK221149A[g]	None	Pre-clinical

Note: For original synthesis, structure and pharmacological properties see the following references:
[a]Williams et al., 1995.
[b]Freidinger and Pettibone, 1997.
[c]Bell et al., 1998.
[d]Ring et al., 2006.
[e]Cirillo et al., 2003; Quattropani et al., 2005.
[f]Serradeil-Le Gal et al., 2004.
[g]McCafferty et al., 2007.

suboptimal pharmacokinetics and oral bioavailability, clinical trials with what appeared to be a promising OT antagonist (L-368,899), (Pettibone et al., 1993; Williams et al., 1995) were discontinued (Freidinger and Pettibone, 1997). Some of the Merck non-peptide OT antagonists, such as L-371, 257 exhibit striking species differences for the V_{1a} receptors in rat and human (Freidinger and Pettibone, 1997). In a recent study, the non-peptide OT antagonist L-368,899 (Pettibone et al., 1993) was reported to have CNS effects after oral administration (Boccia et al., 2007). Another recent study reported that a non-peptide OT antagonist developed at Wyeth-Ayerst, WAY-162720 also penetrates the brain (Ring et al., 2006). Thus the non-peptide OT antagonists L-368,899 and WAY 162720 are promising new tools for studies on the CNS effects of OT. In 1998, Merck reported another promising non-peptide OT antagonist: L-372, 662 (Bell et al., 1998). However, despite promising pre-clinical studies with L-372, 662, the development of tocolytics at Merck has not been resumed. Merck non-peptide OT antagonists, such as L-371,257 (Williams et al., 1995), have proven to be very useful pharmacological tools (see for example Wilson et al., 2001; Hawtin et al., 2005; Liu et al., 2005; Ring et al., 2006).

Currently there are no ongoing clinical trials with non-peptide/OT antagonists. Recently however, three other companies: Serono, Sanofi and GlaxoSmithKline have reported the following promising orally active non-peptide OT antagonists. Serono (Cirillo et al., 2003; Quattropani et al., 2005), Sanofi: SSR126768A (Serradeil-Le Gal et al., 2004) and GlaxoSmithKline: GSK221149A (McCafferty et al., 2007). The GSK221149A non-peptide OT antagonist appears to be a highly promising orally active non-peptide OT antagonist. This molecule exhibits a high affinity ($K_i = 0.65\,nM$) and selectivity for the human OT receptor. It has also been shown to be an effective tocolytic by i.v. and by oral administration in rats. From the data presented by McCafferty et al., 2007, GSK 221149A appears to have a superior spectrum of affinity and selectivity for the human OT receptor versus the $hV_{1a}R$ compared to the non-peptide OT antagonists reported by Merck, Serono and Sanofi. In light of the fact, as noted above, that the two non-peptide OT antagonists L-368,899 and WAY-162720 are brain penetrants (Ring et al., 2006; Boccia et al., 2007), the possibility that non-peptide OT antagonists might cross the blood brain barrier and thus block the central effects of OT both in the foetus and in the mother, will need to be examined before any of these non-peptide OT antagonists are approved for clinical use.

Non-peptide vasopressin agonists

To date, three companies have been active in this area Otsuka, Ferring and Wyeth Ayerst (Table 23). The only non-peptide AVP agonists reported to date are selective for the AVP V_2 receptor. To date, no non-peptide agonists have been reported for the AVP V_{1a} or V_{1b} receptors. In 2000, Otsuka reported the first non-peptide V_2 agonist OPC-51803 (Nakamura et al., 2000; Kondo, 2002). This non-peptide V_2 agonist has been reported to have advantages, in terms of receptor selectivity and oral bioavailability, over the widely used dDAVP (Desmopressin) (Makaryus and McFarlane, 2006) for the treatment of patients with central diabetes insipidus and nocturnal enuresis (Nakamura et al., 2000). This non-peptide V_2 agonist is currently in Phase II clinical development. Also in Phase II clinical development, is the non-peptide V_2 agonist FE-106483 being developed by Ferring. There is no information available on the current status of the trials with both of these non-peptide V_2 agonists: OPC-51803 and FE-106483.

Table 23. Non-peptide V_2 vasopressin agonists

No.	Company	Code	Name	Status
1	Otsuka	OPC-51803[a]	None	Phase II
2	Ferring	FE-106483[b]	None	Pre-clinical
3	Wyeth-Ayerst	WAY 151932 (VNA -932)[c]	None	Pre-clinical

Note: For original synthesis, structure and pharmacological properties see the following references:
[a]Nakamura et al., 2000.
[b]See Ferring web site.
[c]Failli et al., 2006.

Wyeth Ayerst recently reported a novel class of non-peptide V_2 agonists (Failli et al., 2006). Given, that desmopressin has in recent years become widely used in tablet form for oral administration (Lam et al., 1996; Janknegt et al., 1997) and is the current drug of choice for long-term therapy of central diabetes insipidus (Makaryus and McFarlane, 2006), all three non-peptide V_2 agonists face stiff hurdles in displacing the wide acceptance and effective therapeutic use of dDAVP for the treatment of diabetes insipidus. Also, dDAVP, by slow i.v. administration, has for many years been shown to be efficacious in treating mild haemophilia A and von Willebrands disease (for review see Mannucci and Levi, 2007). It remains to be seen whether non-peptide V_2 agonists will be useful therapies for these indications.

Non-peptide oxytocin agonists

This area has seen very little activity over the years (Table 24). However the recent surge in studies showing that OT may be of therapeutic value for the treatment of a wide variety of neuropsychiatric diseases, including, autism, anxiety related disorders and schizophrenia (Insel and Young, 2001; Hollander et al., 2003, 2007; Neumann et al., 2003, 2006; Bosch et al., 2005, 2006; Ebner et al., 2005; Kirsch et al., 2005; Kosfeld et al., 2005; Hammock and Young, 2006; Meinlschmidt and Heim, 2007; Ring et al., 2006), has catalyzed two companies to develop non-peptide agonists for possible therapeutic development. In 2004, Ferring reported a series of non-peptide OT agonists (Pitt et al., 2004). The therapeutic potential of non-peptide OT agonists has been recently reviewed (Ashworth et al., 2006). Very recently, Wyeth-Ayerst obtained a U.S. Patent for the development of therapeutically useful non-peptide OT agonists (Rahman et al., 2007). Studies on a lead compound WAY-267464 were more recently reported (Ring, 2007). Obviously, it is far too early to assess the therapeutic utility of these novel non-peptide OT agonists from Ferring and Wyeth Ayerst.

Table 24. Non-peptide oxytocin agonists

No.	Company	Code	Name	Status
1	Wyeth-Ayerst	WAY-262464[a,b]	None	Pre-clinical
2	Ferring	Compound 27[c]	None	Pre-clinical

Note: For original synthesis, structure and pharmacological properties see the following references:
[a]Rahman et al., 2007.
[b]Ring, 2007.
[c]Pitt et al., 2004.

Research uses of peptide and non-peptide OT and AVP agonists, antagonists, radiolabelled ligands and fluorescent ligands

Peptides as research tools

By virtue of their water solubility, peptides are clearly superior to non-peptides as research tools. Literally, many hundreds of studies have been carried out with peptide agonists, antagonists, radiolabelled and fluorescent ligands for the AVP and OT receptors (see Sawyer et al., 1988; Sawyer and Manning, 1988; Manning and Sawyer, 1989, 1993). The following are examples of highly significant studies carried out with AVP and OT peptide agonists and antagonists during the past 5 years (Loichot et al., 2002; Miller et al., 2002; Pedersen and Boccia, 2002; Chandrashekhar et al., 2003; Liu et al., 2003, 2006; Neumann et al., 2003, 2006; Yu et al., 2003; Andres et al., 2004; Assinder et al., 2004; Derick et al., 2004; Detillion et al., 2004; Oshikawa et al., 2004; Tabrizchi and Ford, 2004; Bosch et al., 2005, 2006; Dobruch et al., 2005; Ebner et al., 2005; Griffante et al., 2005; Hansenne et al., 2005; Hassan et al., 2005; Huber et al., 2005; Ma et al., 2005; O'Donnell et al., 2005; Parker et al., 2005; Reversi et al., 2005; Reymond-Marron et al., 2005, 2006; Cassoni et al., 2006; Condes-Lara et al., 2006; Ferris et al., 2006; Miranda-Cardenas et al., 2006; Ogier et al., 2006; Smith et al., 2006; Volpi et al., 2006; Windle et al., 2006; Yang et al., 2006; Cudnoch-Jedrzejewska et al., 2007; Cumbers et al., 2007; Hagedorn et al., 2007; McKee et al., 2007; Rodrigo et al., 2007; Rojas-Piloni et al., 2007; Subburaju and Aguilera, 2007; Waldherr and Neumann, 2007).

Species differences and lack of specificity

Species differences (Howl et al., 1991; Ruffolo et al., 1991; Pettibone et al., 1992; Thibonnier et al., 1997; Tahara et al., 1999; Andres et al., 2004; Guillon et al., 2004; Chini and Manning, 2007; Chini et al., 2008) and lack of specificity for a given receptor (Manning and Sawyer, 1989, 1993; Chan et al., 2000; Chini et al., 2008) continue to be a concern in this field. We have attempted to address these problems in this review, by presenting the pharmacological properties in rat bioassays and in rat and human receptor assays for many of the most widely used OT and AVP analogues. Both problems and how to deal with them are addressed in more detail in the accompanying publication (Chini et al., 2008). Investigators who purchase these reagents from suppliers are strongly advised to consult the literature to obtain the original pharmacological data. Table 25 lists commercial suppliers of many of the peptides discussed here.

Non-peptides as research tools

Poor aqueous solubility has limited the usefulness of non-peptides as research tools, especially for studies requiring i.c.v. administration. Also, non-peptide antagonists are not available as pharmacological reagents from commercial sources. So they must be obtained from the pharmaceutical companies to which they belong or by resynthesis. For propriety reasons, companies are reluctant to provide samples to other investigators while they are being developed for potential clinical use. So, for these reasons; poor aqueous solubility and lack of availability, non-peptide AVP and OT agonists or antagonists have not been used as widely as research tools as their peptide counterparts. This applies particularly to agonists. Investigators have a wide variety of peptide V_{1a}, V_{1b}, V_2 and OT agonists to chose from, whereas until, very recently, non-peptide agonists did not exist. However, as noted above, non-peptide OT antagonists from Merck, non-peptide V_{1a} and V_2 antagonists from Otsuka and non-peptide V_{1a} antagonists from Sanofi, which are no longer in development have been utilized in a number of important studies. Also, Otsuka and Sanofi have provided their non-peptide V_2 antagonists Tolvaptan (OPC41061) and Satavaptan (SR121463(B)) for use as pharmacological tools in a very recent study which points to the use of V_2 antagonists for the treatment of nephrogenic diabetes insipidus (Robben et al., 2007). Sanofi has also provided the non-peptide V_{1b} antagonist SR 149415 for a very interesting study which demonstrates the use of this non-peptide V_{1b} antagonist as a pharmacological chaperone with the ability to reverse the misfolding of the human pituitary V_{1b} receptor (Robert et al., 2005).

Table 25. Commercial suppliers of peptide OT and AVP agonists, antagonists and radioligands

Company
Peninsula
Bachem
Polypeptide Labs
Sigma Chemical Company
Phoenix Pharmaceuticals
Peptides International
Peptide Institute
Neosystem
New England Nuclear

Therapeutic uses of peptide and non-peptide agonists and antagonists

Peptides as therapeutic agents

OT, Carbetocin, [1-deamino-1-monocarba-2-*O*-methyltyrosine]oxytocin (Su et al., 2007), AVP (Pitressin), dDAVP (Desmopressin) (Minirin) (Makaryus and McFarlane, 2006), Terlipressin (triglycyl lysine vasopressin (Zuberi, 2007)), Felypressin ([2-Phenylalanine]lysine vasopressin) (Cecanho et al., 2006), Lypressin (lysine vasopressin) and the OT antagonist, Atosiban (Akerlund, 2006), have been approved for clinical use in the US, Canada and/or in Europe. For a review of the use of AVP and its analogues in clinical medicine, see Treschan and Peters (2006). As noted above, orally active Desmopressin has become available in tablet form (Lam et al., 1996; Janknegt et al.,

1997). As noted below, this breakthrough is highly relevant to re-energizing the development of peptide V_2/V_{1a} antagonists for therapeutic use as aquaretics.

Non-peptides as therapeutic agents

As noted earlier, to date — only Conivaptan has been approved by the US FDA for i.v. use for the treatment of hyponatremia in a hospital setting (Verbalis, 2006; Walter, 2007). Tolvaptan, Lixivaptan and Satavaptan are currently in clinical trial as orally active therapeutic agents for the treatment of hyponatremia, liver cirrhosis and CHF (Verbalis, 2006). The safety concerns surrounding the use of these Vaptan aquaretics are addressed in a recent review (Verbalis, 2006). In correspondence following the publication of a clinical trial with Tolvaptan (Schrier et al., 2006), all authors express concern that more long-term efficacy and safety studies will need to be carried out with Tolvaptan before it an be recommended as standard therapy for hyponatremia of all aetiologies (Dawwas, 2007; Hoorn and Zietse, 2007; Ozkan et al., 2007; Weise et al., 2007). Clearly further studies are needed to determine best practice clinical use over time of these long awaited therapeutic agents.

Conclusion

While OT and AVP agonists, antagonists, radiolabelled and fluorescent ligands have found widespread use as research tools in a wide variety of in vitro and in vivo studies on the peripheral and central effects of these peptides, many investigators continue to use some earlier peptides which lack specificity for a given receptor type.

In this review we have attempted to provide a snapshot of the current state of the art on the available OT and AVP agonists and antagonists for the OT receptor, for the V_{1a}, V_{1b} and V_2 receptors and for the putative AVP hypotensive (V_{1c}?) receptor. Data on the most widely used and some very new OT and AVP agonists are presented in Tables 1–8. Tables 4–7 contain rat bioassay data and human receptor data on recently discovered high affinity, selective V_{1b} receptor agonists. These promise to be very valuable new pharmacological tools for studies on the rat and human V_{1b} receptors. Species differences between the rat and human V_2 receptors are clearly evident in the data presented in Tables 4 and 5. The findings reported here have profound implications for previously published AVP antidiuretic agonists. In all likelihood, many will be found to exhibit potent V_{1b} agonism in the rat and in the human — similar to that observed for dDAVP (Saito et al., 1997) and for the selective hV_{1b} receptor agonists which we reported (Guillon et al., 2006). The novel hypotensive agonists reported in Table 8 are not yet available commercially. Here we point to the shortcomings of many of our most potent V_{1a} antagonists, such as $d(CH_2)_5[Tyr(Me)^2]AVP$ (Kruszynski et al., 1980). The V_{1a} antagonists that have been examined in anti-oxytocic (anti-OT) assays, exhibit OT antagonism. Unfortunately, many of the original V_{1a} antagonists were not examined in anti-OT assays. However, to be on the safe side, investigators should assume that they will exhibit some anti-oxytocic potency in vitro and possibility also in vivo. The selective V_{1a}/OT antagonists in Table 10 are not yet available from commercial sources. While Tables 11 and 12 list some non-selective V_{1b}/V_{1a} antagonists — none are really ideal. So the search for selective peptide V_{1b} antagonists must continue. Caution also needs to be exercised in the use of the selective cyclic and non-selective V_2/V_{1a} antagonists in Table 13. These are selective for the V_2 receptor with respect to the V_{1a} receptor in the rat. They are not selective with respect to the OT receptor. Since their V_{1b} receptor properties have not been examined, some of these may also not be selective with respect to the V_{1b} receptor. Species differences between the rat and human V_2 receptors, proved to be a major stumbling block to the development of a peptide V_2 antagonist which would be an effective aquaretic in humans (Ruffolo et al., 1991; Manning et al., 1995b; Thibonnier et al., 1997). The design of a peptide V_2 antagonist which is an effective aquaretic in humans remains a challenging goal. Peptide V_2/V_{1a} antagonists which are effective in humans would be very valuable pharmacological tools

and analogous to the US FDA approved Conivaptan, could also be excellent candidates for development as therapeutic agents by i.v. administration in a hospital setting. As illustrated by the pharmacological properties of the peptide OT antagonists in Tables 15 and 16, striking progress has been made in the development of selective OT antagonists for the rat and for the human receptor.

Some widely used and more recent radiolabelled and fluorescent ligands for the OT and AVP V_{1a} and V_2 receptors are listed in Tables 17–19. The tritiated non-peptide V_{1b} antagonist, SR-149415 (Serradeil-Le Gal et al., 2007) promises to be a useful new tool for studies on rat and human V_{1b} receptors. Finally, we report some preliminary findings on promising new bivalent antagonist ligands for the AVP hV_{1a} and hOT receptors (Chini et al., 2007). While the bivalent ligand approach has proved to be very valuable for a wide variety of GPCR ligands (Halazy, 1999), with the exception of a study by Howl et al. (1997), until now, this approach has been unexplored in the AVP/OT receptor field. The preliminary findings reported in Table 20 clearly indicates the merits of further exploration of this approach for the design of OT and AVP receptor selective agonists and antagonists.

In this review we have also presented a summary in Tables 21–24 of the current state of pre-clinical and clinical development of non-peptide AVP V_{1a}, V_{1b}, V_2/V_{1a} antagonists, non-peptide OT antagonists and non-peptide OT and AVP V_2 agonists. To date, there are no non-peptide V_{1a} or OT antagonists currently in clinical trial. However, the recently reported V_{1a} antagonist SRX251, reported by Azevan (Ferris et al., 2006), offers promise as a new therapeutic agent for the treatment of aggression and for anger management. The non-peptide V_{1b} antagonists SSR-149415 and ORG 52186 from Sanofi (Serradeil Le-Gal, 2002a, b, 2007) and Organon (Letourneau et al., 2006; Craighead and MacSweeney, 2008) respectively are currently in pre-clinical development for the treatment of anxiety and depression. The mixed V_2/V_{1a} non-peptide antagonist, Conivaptan (Vaprisol) was approved by the US FDA for i.v. use only in a hospitalized setting for the treatment of hyponatremia (Walter, 2007). The three non-peptide V_2 antagonists (Vaptans), currently in clinical trial: Tolvaptan, Lixivaptan and Satavaptan (Verbalis, 2006) have not yet received FDA approval. The non-peptide V_2 agonists and non-peptide OT agonists currently in pre-clinical development may offer advantages over desmopressin and OT in certain clinical situations. The results of eventual clinical trials for these non-peptide agonists will be awaited with much interest.

On a somewhat "back to the future" note, it may be fitting to recall the role that peptides have played in the development, "from laboratory to the bedside", of AVP V_2 receptor antidiuretic antagonists (Vaptans). This has been a long saga. The first AVP antidiuretic V_2 receptor antagonists, reported over 25 years ago, were peptides (Sawyer et al., 1981). This breakthrough gave rise to the expectation that it would only be a matter of time until a peptide V_2 antagonist would be developed as an aquaretic for the treatment of hyponatremia due to SIADH (Kinter et al., 1988). Studies carried out in our laboratories and in the Smith Kline French laboratory led to two highly promising candidates (Manning et al., 1984; Kinter et al., 1988). However, in pre-clinical studies with human volunteers, these were found to be agonists (Ruffolo et al., 1991). This highly unexpected finding, combined with the discovery in the Otsuka Laboratory of non-peptide V_{1a} and V_2 antagonists (Yamamura et al., 1991, 1992) led to the rapid cessation of any further attempts to develop peptide based V_2 antagonists for therapeutic use.

Now may be the time to take another look at peptide V_2 antagonists as new "old" therapeutic agents for the treatment of hyponatremia. As noted above, the non-peptide V_2/V_{1a} antagonist Conivaptan (Vaprisol), approved for clinical use i.v., is not suitable for standard therapy for SIADH. (See PBM drug monograph entitled "Conivaptan hydrochloride injection (Vaprisol®)" on www.pbm.va.gov). Likewise, the three non-peptide V_2 antagonists: Tolvaptan, Satavaptan and Lixivaptan also possess serious shortcomings in terms of their efficacy and safety as routine therapeutic agents for the treatment of SIADH

and CHF. The correspondence following the SALT trial on Tolvaptan (Schrier et al., 2006) express serious concern on these issues (Dawwas, 2007; Hoorn and Zietse, 2007; Ozkan et al., 2007; Weise et al., 2007).

The recent dramatic increase in the number of peptides which have become available for therapeutic use (Verlander, 2005), including the peptide OT antagonist Atosiban (Akerlund, 2006), combined with striking advances in peptide delivery methods (see for example, Mehta, 2004; Malik et al., 2007; Mastrandrea and Quattrin, 2006; Purcell et al., 2007), provide compelling reasons to re-investigate peptide V_2 and V_2/V_{1a} antagonists for development as potential aquaretic agents for the treatment of hyponatremia. Also, as noted above, dDAVP (Desmopressin, Minirin), for many years used solely as a nasal spray, is now available in oral form. Orally active Desmopressin has been shown to be both safe and highly efficacious, (Lam et al., 1996; Janknegt et al., 1997) and is widely accepted by clinicians as the drug of choice for treating central diabetes insipidus (Makaryus and McFarlane, 2006). This breakthrough in the development of the peptide V_2 agonist, dDAVP for oral administration provides further convincing evidence that peptide V_2 antagonists could likewise be developed as effective and safe aquaretics for oral administration. Our discovery of the first constitutively active human V_2 receptor, which can discriminate between V_2 antagonists that exhibit partial V_2 agonism and V_2 antagonists that are devoid of V_2 agonism (Morin et al., 1998), should greatly facilitate the design of peptide V_2 antagonists which are effective aquaretics in humans.

Finally, from all of the foregoing, it is abundantly clear that striking progress has been made in the design of peptide and non-peptide OT and AVP agonists, antagonists, and their radiolabelled, fluorescent labelled and bivalent ligands for use as research tools and therapeutic agents. However, as also noted above, species differences and the lack of single V_{1a}, V_{1b}, V_2 or OT receptor specificity continue to pose serious and intriguing design challenges. So in a nutshell, there is still much work to be done and many more exciting discoveries to be made in this field, especially in the areas of coupling specific ligands (Reversi et al., 2005; Chini and Manning, 2007) and bifunctional ligands (Chini et al., 2007).

Acknowledgements

Work from the authors' laboratories was supported in part by research grants from the National Institute of General Medical Sciences (No. GM-25280: Maurice Manning), The Italian Association for Cancer Research (AIRC 2006) and the Carpilo Foundation (Grant 200610882) (Bice Chini), INSERM, Institute de la Santé et de la Recherche Médicale and CNRS (Centre National de la Recherche Scientifique) (Thierry Durroux; Bernard Mouillac; Gilles Guillon). We are greatly indebted to our former colleagues and co-workers who have participated over the years in the published studies cited here, especially Dr. Ling Ling Cheng, Dr. Hazel H. Szeto and Ms. Becky Wo for their invaluable recent contributions to these studies. In particular, we thank Claude Barberis for the previously unpublished human receptor data for HO[Thr^4]OT (footnote to Table 1) and for $d(CH_2)_5[Tyr(Me)^2]OVT$ (Table 16), Dr. Rainer Landgraf for his helpful comments on the relative merits of peptides and non-peptides as research tools and for editing this manuscript and Ms. Ann Chlebowski for her expert assistance in the preparation of the manuscript.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 38

Affinity and efficacy of selective agonists and antagonists for vasopressin and oxytocin receptors: an "easy guide" to receptor pharmacology

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Abstract: The development of "selective" drugs targeting oxytocin/vasopressin receptors has enormously progressed since the original synthesis of oxytocin more than 50 years ago. However, several factors still hamper the availability of a rich and complete range of selective agonists and antagonists acting at the different oxytocin/vasopressin receptor subtypes, making the use of these drugs still a daunting task. In this paper we will briefly review the major problems encountered when dealing with oxytocin/vasopressin selective ligands, proving few rules for their correct pharmacological use, in order to avoid common pitfalls. Finally, we will glimpse at new challenges, such us the discovery of coupling selective ligands, which foster the search for new classes of selective compounds.

Keywords: vasopressin; oxytocin; agonist; antagonist; selectivity; species difference

The design and use of selective agonists and antagonists acting at the oxytocin/vasopressin (OT/AVP) receptors has been and continues to be a difficult task for two principal reasons:

- The high sequence homology among the AVP and OT $V_{1a}/V_{1b}/V_2$ and OT receptor subtypes and the high degree of chemical similarity among the active ligands often results in overlapping selectivity profiles.
- The presence of subtle differences among receptor sequences in various animal species are responsible for important changes in the selectivity profile of some ligands; for this reason, the pharmacological data obtained in one species cannot be extrapolated "tout court" to other species.

Here we propose a quick guide to the use of "selected" useful drugs together with few key principles to be followed to design and perform pharmacological assays on OT/AVP receptors. We will not consider here the use of selective analogues in in vivo bioassays, as this is the subject of an extensive review that also appears in this issue, to which we refer for a historical and critical evaluation of the different analogues (Manning et al., 2008).

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DOI: 10.1016/S0079-6123(08)00438-X

Let us first discuss the operational definition of a ‘‘selective’’ ligand. In current pharmacological terms, a selective ligand is defined on the basis of either its high affinity or biological activity on a specific receptor when compared to the others (Kenakin, 2003). However, it is to be kept in mind that all these structurally related analogues lack an *absolute* receptor subtype selectivity; their receptor subtype selectivity is always relative and concentration dependent. As selectivity is defined on the basis of the capability of a single analogue to bind to and/or activate a single OT/AVP receptor subtype, it is of course an absolute requirement for these analogues to have been tested on *all* the OT/AVP receptor subtypes within the species under investigation. Unfortunately, pharmacological data are often partial and, for several analogues, the full characterization on all the OT/AVP receptor subtypes is missing, making their use as ‘‘selective’’ analogues impossible.

Selectivity may be defined on the basis of binding affinity of the ligand. In this case, because the affinity of the natural ligands, OT or AVP, defined by their affinity constants (K_d), is in the nanomolar range, a good ligand should possess an affinity, again defined by its affinity constant (K_d or K_i), in the same range. To be ‘‘selective’’ on one receptor subtype, its affinity constant on the other three members of the family should be at least *two orders of magnitude* higher. Using these very tight criteria, the most selective ligands for the human and the rat OT/AVP receptors are listed in Fig. 1 (Ahrabi et al., 2007; Guillon et al., 2004; MacSweeney et al., 2007; Murat et al., 2007; Pena et al., 2007; Serradeil-Le Gal et al., 2004, 2007).

A crucial issue concerning the use of these analogues is their availability; as some of these

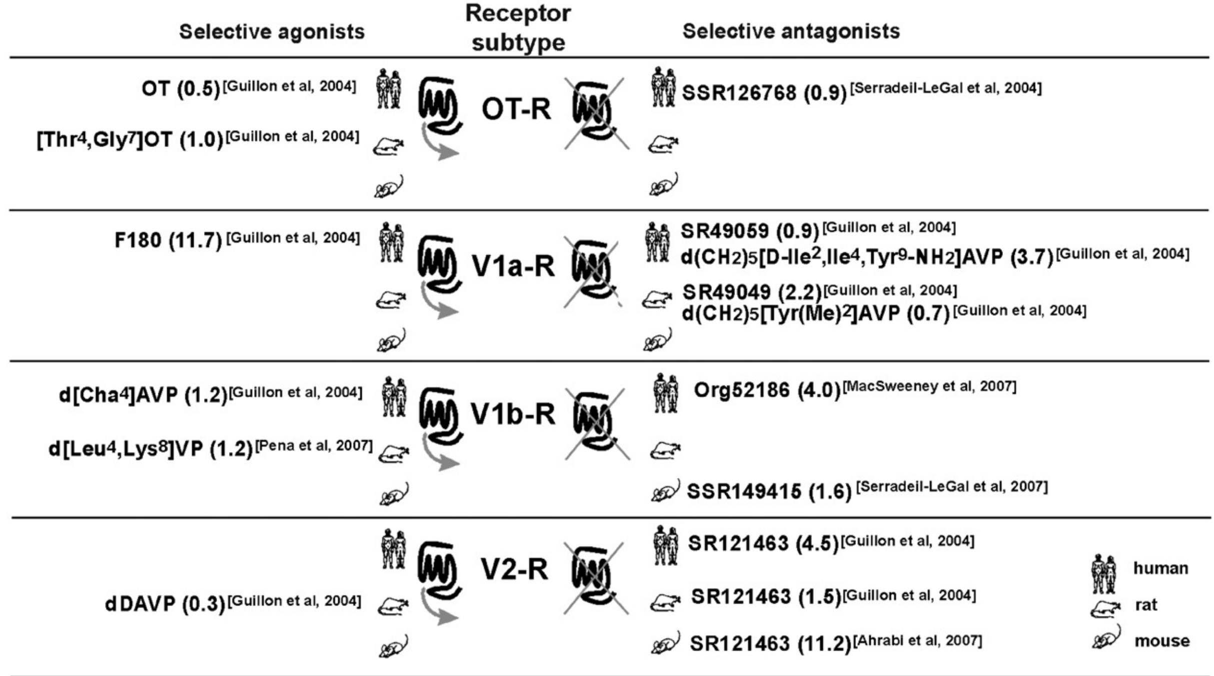

Fig. 1. Highly selective receptor subtype OT/AVP ligands. To be included in this figure, a selective ligand fulfilled the following pharmacological criteria: (1) to have been tested on *all* the OT/AVP receptor subtypes (OTR, V_{1a}, V_{1b}, V_2) present in a single species, (2) to possess an affinity (defined by its affinity constants K_d or K_i) in the nanomolar range for the receptor subtype it is specific for and (3) to possess an affinity constant at least two orders of magnitude higher for the three other receptor subtypes. The affinity constant of the analogue for its selective receptor subtype, expressed in nanomolar, is reported in round brackets with the appropriate reference in square brackets.

analogues are not commercially available, restrictions in their supply would greatly hamper further scientific advancements in the field.

Another important point that clearly emerges from Fig. 1 is the lack of data concerning ligand selectivity in mice. Despite the huge relevance in many areas of basic and preclinical science of genetically modified OT/AVP peptide/receptors in mice, not many analogues have been extensively tested in this species, a gap that certainly needs to be filled.

We would also comment on three analogues that have been extensively used in the past as ''selective'' compounds and that have recently turned out to be less selective than previously thought. First, dDAVP is rather unspecific in humans, where it is a mixed V_2/V_{1b} agonist (see Table 4 in Manning et al., 2008). The ''Manning compound'' is not selective for the V_{1a} receptor as it also binds to the OTR (see Table 10 in Manning et al., 2008). Finally, Atosiban, used as a tocolytic agent in humans, is a mixed V_{1a}/OT receptor antagonist (see Tables 15 and 16 in Manning et al., 2008); furthermore, this analogue has been recently shown to be an antagonist at the OT receptor/Gαq coupling but an agonist at the OTR/Gαi coupling, thus belonging to a new class of compounds known as ''biased agonists'' or ''functional agonists'' (Reversi et al., 2005). The relevance of coupling-selective analogues is due to their capability to selectively activate only one signalling pathway of the many that a single receptor may activate, thanks to its promiscuous coupling to several different G-proteins and to other signalling intermediates (Urban et al., 2007). This is particularly relevant as, in some cases, the signalling pathways activated by a single G-protein-coupled receptor may act synergistically, but they may also give rise to opposite effects on the same cellular function. Developing coupling-selective analogues represents a new challenge in molecular pharmacology, because they will activate, for any single receptor subtype, only one downstream signalling pathway, representing a class of compounds highly selective at the single receptor level.

Let us finally discuss a few practical cases in which binding or functional assays are used to demonstrate and characterize OT/AVP receptors in biological samples.

Binding assays

The first basic question that often needs to be addressed is whether OT/AVP receptors are present in the sample (cell culture, tissue, organ) under investigation. To answer to this question, we suggest to perform binding assays using the tritiated natural agonists, [^{3}H]AVP and [^{3}H]OT, which are commercially available. In particular, [^{3}H]AVP presents the advantage of binding with similar affinities to $V_{1a}/V_{1b}/V_2$ and OTR in all the mammalian species investigated so far, allowing the simultaneous labelling and identification of all the AVP/OT receptors present in the sample. An incubation time of 60 min at 30°C or 37°C is usually enough to reach the equilibrium; however, if the assay is performed on intact cells, lower temperatures are necessary to avoid labelled hormone internalization, and thus longer incubation times may be required (up to 4 h at 4°C).

It may then be necessary to further characterize the receptor subtype(s) by means of binding experiments. To this aim, the best procedure is to perform competition binding experiments, in which the receptors labelled with a fixed concentration of [^{3}H]AVP (usually 1–5 nM) are incubated in the presence of increasing concentrations of unlabelled selective analogues such as those listed in Fig. 1. Because all of the analogues listed in this figure are selective for a given VP/OT receptor isoform and exhibit a nanomolar affinity for the receptor isoform they are specific for, a concentration of 30 nM of any selective analogue should completely inhibit the specific [^{3}H]AVP binding on its specific receptor subtype. In contrast, because they have an affinity at least 100-fold higher for the three other AVP/OT subtypes, the same concentration of 30 nM should only weakly inhibit [^{3}H]AVP binding on the receptor subtypes they are not specific for.

Second messengers or functional studies on membranes, cells or tissues sections

For functional tests, selective agonists are always preferred (if available) because experiments are easier to perform and to analyse. When

experiments are performed on membrane homogenates or on cells in culture, a single dose of the selective agonist at a concentration around its K_d/K_i (generally between 1 nM and 10 nM) may initially be used. If a significant response to this concentration is obtained, dose-response curves must be performed. The K_{act} obtained for this selective agonist (concentration of agonist leading to half maximal activity) should be around its K_d/K_i. Furthermore, to unambiguously determine the AVP/OT receptor isoform involved, it is necessary to show that other agonists, selective for the other receptor subtypes, when used at their appropriate concentrations (around their K_d/K_i values), are not effective. If working on acute slices of living tissues, this concentration will probably need to be increased to allow the diffusion of the analogue within the tissues. However, special care should be taken in order to keep the selectivity of the peptide, because at concentrations 50–100-fold over its K_d/K_i, the agonist specificity of the agonist may be lost. Alternatively, selective antagonists may be used to abolish the agonist-induced effect. When using a "selective" antagonist to compete for a "selective" agonist, always follow the same rule of not working at a concentration exceeding 10–50-fold its K_i/K_d, as antagonists also usually present a two-log selectivity range in which they are selective.

If no selective agonist is available, a selective antagonist can be used in more complex experiments. First, the lowest concentration of AVP/OT leading to a clear but not maximal stimulation should be determined (a nanomolar concentration is generally sufficient for experiments performed on cells or membranes, a 10–30 nM concentration for experiments performed on tissues slices). Then, samples are pre-incubated for 15 min at 37°C with increasing amounts of a selective antagonist (at concentrations ranging between 1–100 its K_d/K_i), followed by AVP/OT stimulation at the concentration determined above. If a clear inhibition of AVP/OT-stimulated response is obtained with a concentration of a selective antagonist at a concentration approximately 10-fold its K_d/K_i, the antagonist used identifies the receptor subtype. The rational for choosing the concentration of a selective agonist or antagonist in the experiments described above is based on the existence of an excellent correlation between binding parameter of a given analogue (K_d) and its ability either to stimulate or inhibit the functional response measured (Cheng et al., 2004).

Conclusions

Far from being exhaustive, we hope to have provided some general rules to assist with the choice and use of OT/AVP ligands, with the overall aim being to help in avoiding pitfalls when setting up the most common pharmacological assays. Please refer to our paper in this issue (Manning et al., 2008) for an extensive review of the OT/AVP analogues developed through the years, as well as for detailed discussion of their selectivity profiles in in vivo bioassays. Finally, further data on receptor and ligand selectivity can be found at the IUPHAR (The International Union of Basic and Clinical Pharmacology) Database on Receptor Nomenclature and Drug Classification (http://www.iuphar-db.org).

Acknowledgements

This work was supported in part by research grants from the National Institute of General Medical Sciences (No. GM-25280; Maurice Manning), The Italian Association for Cancer Research (AIRC 2006; Bice Chini), Institute de la Santé et de la Recherche Médicale (INSERM) and Centre National de la Recherche Scientifique (CNRS; Gilles Guillon).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 39

Prevention of hypoxic brain oedema by the administration of vasopressin receptor antagonist OPC-31260

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Abstract: The numerous situations which can result in cerebral hypoxic damage occur in newborn infants and in the elderly. In research aimed at more effective therapeutic intervention in ischaemic disorders of the brain, the animal model used and the principles of the causal therapy should be better outlined. The effects of the non-peptide AVPR (V2) antagonist 5-dimethylamino-1-[4-(2-methylbenzoylamino) benzoyl]-2,3,4,5-tetrahydro-1*H*-benzazepine hydrochloride (OPC-31260) on the cerebral oedema induced by general cerebral hypoxia were studied in rats. The general cerebral hypoxia was produced by bilateral common carotid ligation in Sprague–Dawley rats of the CFY strain. By 6 h after the ligation, half of the rats had died, but the survival rate was significantly higher following OPC-31260 administration. Electron microscopic examinations revealed typical ischaemic changes after the carotid ligation, and OPC-31260 treatment did not significantly reduce the hypoxic signs in the brain cortex; only a certain decrease in the pericapillary oedema was observed. The carotid ligation increased the brain contents of water and Na^+ and enhanced the plasma AVP level. The increased brain water and Na^+ accumulation was prevented by OPC-31260 administration, but the plasma AVP level was further enhanced by OPC-31260. These results demonstrate the important role of AVP in the development of the disturbances in brain water and electrolyte balance in response to general cerebral hypoxia. The carotid ligation-induced cerebral oedema was significantly reduced following oral OPC-31260 administration. The protective mechanism exerted by OPC-31260 stems from its influence on the renal AVPR (V2). These observations might suggest an effective approach to the treatment of global hypoxia-induced cerebral oedema in humans.

Keywords: carotid ligation; cerebral oedema; hyponatraemia; vasopressin receptor antagonist; non-peptide

Introduction

Yamamura et al. (1992) reported a detailed characterization of an orally effective, non-peptide

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DOI: 10.1016/S0079-6123(08)00439-1

AVPR (V2) antagonist, 5-dimethylamino-1-[4-(2-methylbenzoylamino)benzoyl]-2,3,4,5-tetrahydro-1*H*-benzazepine hydrochloride (OPC-31260), which blocks the binding of AVP to renal plasma membranes in vitro, inducing a substantial diuretic effect. The urinary output and osmolality following oral administration of 30 mg/kg OPC-31260 were measured in rat. The duration of the aquaretic effect of OPC-31260 proved to be about 6–8 h (Laszlo et al., 1999). Other authors have described a considerable aquaretic effect of OPC-31260 following intravenous or oral administration to healthy subjects (Ohnishi et al., 1993, 1995; Shimizu, 1995). OPC-31260 appears to be a promising drug from the viewpoint of clinical practice (Yamamura et al., 1993; Okada et al., 1994; Tsuboi et al., 1994a, b; Fujita et al., 1995). We therefore set out to investigate whether the non-peptide AVPR (V2) antagonist OPC-31260 can prevent the development of cerebral oedema following bilateral carotid artery occlusion. A further aim was to study the role of AVP in the pathomechanism of cerebral oedema observable after cerebral hypoxia, and the mode of action of OPC-31260 in global cerebral ischaemia. We used Sprague–Dawley rats of the CFY strain. Earlier, characteristic symptoms of global cerebral ischaemia were described in this strain following bilateral carotid artery occlusion (Koltai et al., 1984; Tosaki et al., 1985).

Methods

Experimental protocol

The experiments were performed on 3- to 5-month-old male CFY rats, ranging in weight from 200 to 280 g (bred in our animal house; breeding stock from the Laboratory Animals Producing Institute, Gödöllõ, Hungary). The animal care and research protocols were in accordance with the guidelines of our university. The animals were subjected to ether anaesthesia during operations. The rectal temperature was monitored, and cooling was prevented with an electric heating pad. Bilateral ligation of the common carotid arteries was performed for 1, 4 and 6 h under ether anaesthesia. In the control, sham-operated groups, the surgical manipulation was the same, but without carotid ligation. OPC-31260 in a dose of 30 mg/kg, or vehicle only, was administered by gastric tube: OPC-31260 was dissolved in water (15 mg/ml) immediately after the carotid ligation. The dose–response curve for OPC-31260 was reported by Yamamura et al. (1993). The antagonist dose and the duration of its effect were described in an earlier publication (Laszlo et al., 1999). At the end of the experiments, the rats were killed by decapitation under ether anaesthesia.

Blood pressure measurement

In a separate group of animals (20 rats), the mean arterial blood pressure was measured. Ten rats underwent only bilateral carotid ligation, while the other group (10 rats) was treated orally with OPC-31260 (30 mg/kg) immediately after the ligation. Under ether anaesthesia, a polyethylene tube was inserted into the right carotid artery and diluted heparin was injected as an anticoagulant. Blood gases were monitored. The carotid cannulae were connected to Statham P23D transducers and blood pressure was recorded continuously during 6 h with a Hellige recorder. The method was described in detail earlier (Laszlo et al., 1995).

Brain water and electrolyte contents

The brain water content was determined by dehydration to weight constancy; 1, 4 or 6 h after the operation, the brain was removed and weighed before and after drying at 200°C for 24 h. This was followed by ashing at 550°C for 20 h, after which the ash was dissolved in 5 ml of 3 mM HNO_3 and the resulting solution was diluted 10-fold with deionized water. With a Perkin-Elmer 306 atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) and use of an air–acetylene flame, the Na^+ content was determined to be 330.3 nM and the K^+ content to be 404.4 nM. The slit width was 0.7 and 2 mm, respectively. Tap water (0.5 ml) was administered instead of OPC-31260 to the control animals. The brain Na^+ and K^+ determinations were carried out in the Central Research Laboratory, Medical University, Szeged, Hungary. The plasma Na^+ and K^+ levels were

determined with a flame-photometric micro method, and the osmolality with an Advance osmometer in 10 rats.

Electron microscopic examination

Following ether anaesthesia, the animals were killed by decapitation 1, 4 or 6 h after the carotid ligation. The brains were immediately removed and small pieces of the parietal cortex were placed in the fixative solution (1% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) for 4 h at 4°C. Before the electron microscopic study, light microscopic examination was carried out after staining with methylene blue-Azure II. After overnight washing in 0.1 M phosphate buffer, postfixation was performed in phosphate-buffered (pH 7.4) 1% osmium tetroxide solution for 1 h, and the tissue samples were dehydrated in a graded series of increasing ethanol concentration before embedding in Spurr. During dehydration, they were stained en bloc with uranyl acetate 0.5% (w/v) in 70% ethanol for 15 min. Ultrathin sections were cut on a Reichert–Jung Ultracut E ultramicrotome, contrasted with lead citrate and examined in a Zeiss EM 902 electron microscope. We used six rats in each group for light or electron microscopic examination. The evaluation was performed blind.

Plasma AVP determination

The plasma AVP levels were measured by RIA, based on a technique described by Dogterom et al. (1978) with some modifications, as reported in detail earlier (Jojart et al., 1987; Laczi et al., 1987). Plasma samples were stored at −20°C until assaying. RIA was performed within 72 h after sampling. AVP extraction was carried out with an Amprep C8 minicolumn (code RPN 1902 Amersham, Buckinghamshire, UK). The sensitivity of the RIA was 1 pg per assay tube. AVP levels are given in pg/ml plasma.

Statistical analysis

The data are expressed as means ± S.E.M. of the results for the total number of rats per experimental group. Statistical analysis was performed by using the Mann–Whitney non-parallel *U*-test, the Bonferroni multiple comparisons test and the Fisher exact test, where appropriate. *P* values less than 0.05 were considered significantly different.

Results

The survival rate proved to be significantly higher in the OPC-31260-treated rats 4 or 6 h after the carotid ligation (Table 1). During the observation for 1, 4 or 6 h, the plasma osmolality and the plasma Na^+ and K^+ levels did not change significantly following ligation, and these data are therefore not reported here. Immediately after the ligation, the blood pressure significantly increased, but then returned to the control level (basal: 109.0 ± 6.2*, 15 s: 137.3 ± 9.7, 10 min: 124.5 ± 7.1, 1 h: 124.3 ± 7.4, 4 h: 125.1 ± 9.4, 6 h: 102.3 ± 8.7 Hgmm, $n = 10$, *S.E.M.).

Table 1. Survival rates after bilateral carotid ligation and OPC-31260 administration

Groups	Number of animals	Hours after ligation	Number of surviving animals
1. Control untreated	15	–	15
2. Control + OPC-31260	15	–	15
3. Carotid ligation	15	1	13
4. Carotid ligation + OPC-31260	18	1	18
5. Carotid ligation	18	4	10*
6. Carotid ligation + OPC-31260	20	4	18+
7. Carotid ligation	21	6	8*
8. Carotid ligation + OPC-31260	20	6	15+

Note: Higher survival rates were observed in the OPC-31260-treated rats 4 and 6 h after the carotid ligation. The level of statistical significance is (*) $P < 0.05$ as compared with the control group (1), and (+) $P < 0.05$ as compared with the untreated ligated groups (3, 5 and 7).

The blood pressure did not change following OPC-31260 administration in comparison with the non-treated ligated group. The brain water content and ion concentrations are given in Table 2. These were not changed 1 h after carotid ligation and this was not modified by OPC-31260 administration. Four or 6 h following carotid ligation, the brain water content was significantly increased and the Na^+ concentration was enhanced in parallel, but the K^+ level remained normal. The increases in the brain water and Na^+ levels were prevented by the simultaneous administration of OPC-31260.

No changes were detected in the brain structure by light microscopic examination at any time during the whole experimental period (1, 4 and 6 h after the ligation) and electron microscopy did not reveal any morphological alterations in the structure of the capillaries 1 h after the ligation. Similarly as in the control animals, unaltered endothelial cells, astrocytic end-feet and basal lamina were found (Fig. 1A). Six hours after the carotid occlusion, however, the morphological signs of the blood–brain barrier damage were obvious; the capillaries were surrounded by swollen astrocytic processes (Fig. 1B). At that time point, moderate swelling of the mitochondria was observed in the neuronal somata and processes, as an indication of the development of hypoxia in the cortical cells. OPC-31260 administration did not result in any significant reduction in the structural damage; the astrocytic swelling was still obvious in animals ligated for 6 h and treated with the drug (Fig. 1C).

The plasma AVP level was increased 1 h after carotid ligation (Fig. 2). The enhancement of the plasma AVP concentration was more pronounced 4 or 6 h following ligation. OPC-31260 significantly increased the AVP level in the sham-operated control rats. After the common carotid ligation, OPC-31260 administration elicited a further increase in plasma AVP level, depending on the time that had elapsed after the ligation.

Discussion

The present results demonstrate that a high level of AVP and a significant degree of cerebral oedema develop following bilateral carotid ligation. The increase in the AVP may originate as a result of hypoxic stress, and the elevated AVP concentration may play a significant role in the development of cerebral oedema, influencing the brain capillary permeability and the water metabolism.

It appeared very important to study the different modes of prevention of cerebral oedema. As regards the protective mechanism of OPC-31260,

Table 2. Brain water content and ion concentrations following bilateral carotid artery ligation and OPC-31260 administration

Groups	Number of animals	Hours after ligation	Water content (g/100 g wet brain)	Ion concentration (mmol/kg dry brain weight)	
				Na^+	K^+
1. Control untreated	10		77.52 ± 0.3	281.0 ± 12.3	342.9 ± 12.3
2. Control + OPC-31260	12		77.21 ± 0.2	278.3 ± 11.8	350.5 ± 10.6
3. Carotid ligation	9	1	77.85 ± 0.3	290.5 ± 16.1	340.8 ± 16.1
4. Carotid ligation + OPC-31260	12	1	77.53 ± 0.3	275.7 ± 15.0	337.3 ± 14.9
5. Carotid ligation	7	4	79.51 ± 0.4*	340.9 ± 10.2*	341.7 ± 18.3
6. Carotid ligation + OPC-31260	12	4	77.44 ± 0.2$^+$	276.6 ± 11.8$^+$	339.1 ± 11.8
7. Carotid ligation	6	6	79.75 ± 0.3*	353.1 ± 14.5*	337.5 ± 16.3
8. Carotid ligation + OPC-31260	10	6	77.37 ± 0.3$^+$	283.0 ± 13.6$^+$	341.9 ± 15.7

Note: OPC-31260 was administered by gastric tube in a dose of 30 mg/kg immediately after carotid ligation. OPC-31260 did not alter the brain water content and ion concentrations in the control rats (Group 2). One hour after carotid ligation (Group 3), the brain water content and ion concentrations were unchanged and this was not modified by OPC-31260 administration (Group 4). Four (Group 5) and 6 h (Group 7) following carotid occlusion, the brain water content and Na^+ concentration were significantly increased. The increases in the brain water and Na^+ content were prevented by the simultaneous administration of OPC-31260 (Groups 6 and 8). Results are given as mean values ± S.E.M., where the level of statistical significance is (*) $P < 0.05$ as compared with the control group (1), and ($^+$) $P < 0.05$ as compared with the untreated groups (5 and 7).

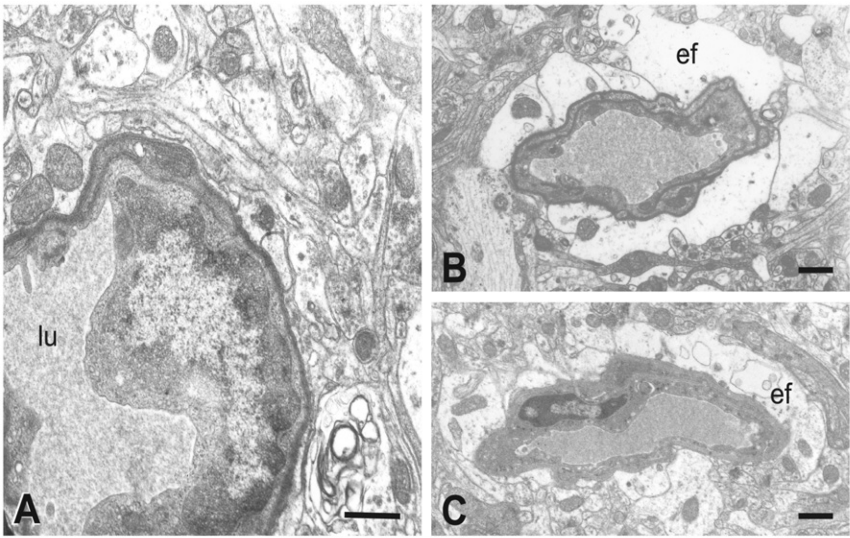

Fig. 1. Electron microscopic changes in cerebral cortical capillaries after carotid occlusion and OPC-31260 treatment. As compared with the control animals (A), 6-h cerebral ischaemia (B) resulted in ultrastructural damage; electron microscopy revealed astrocyte end-foot process oedema (ef) and vacuolization. AVPR (V2) antagonist OPC-31260 (C) treatment did not significantly reduce the extent of the blood–brain barrier injury. lu: capillary lumen; bar: 1 μm.

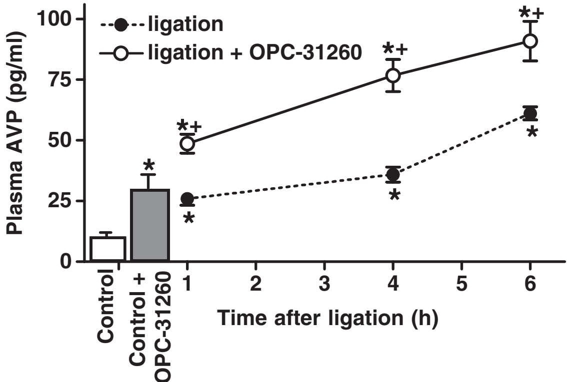

Fig. 2. Plasma AVP level following bilateral carotid artery ligation and treatment with OPC-31260. The AVP levels were determined 1, 4 and 6 h after the ligation. OPC-31260 was administered orally immediately after the carotid ligation (30 mg/kg). The plasma AVP level was increased 1 h after ligation. The plasma AVP concentration enhancements were more pronounced 4 and 6 h following ligation. OPC-31260 significantly increased the AVP level in the sham-operated control rats. After the common carotid ligation, OPC-31260 administration elicited a further increase in plasma AVP level, depending on the time that had elapsed after the ligation. Data are shown as means ± S.E.M. for 10 rats in each group, where the level of statistical significance is (*) $P<0.05$ as compared with the control group, and ($^{+}$) $P<0.05$ as compared with the OPC-31260-treated group.

at least four possibilities have to be considered: (1) Blockade of the increased AVP release following carotid ligation. (2) A decrease of the ischaemia following carotid ligation. (3) A decrease in brain capillary permeability (a direct effect). (4) An effect on the renal tubule function (an indirect diuretic effect). (1) The first of these hypotheses is unacceptable, since the present study revealed that OPC-31260 did not inhibit AVP release: it increased the AVP levels both in the controls and in the rats which underwent carotid ligation. Similar observations were made in earlier experiments: The V_2 receptor antagonists increase the plasma AVP concentration (Laszlo et al., 1999; Decaux, 2001; Guyader et al., 2002; Wong et al., 2003). It should be mentioned here that the mechanism of plasma AVP enhancement following OPC-31260 administration is unknown. The longer biological half-life of AVP after OPC-31260 administration may play a role in the elevation of the plasma AVP level (Molnar et al., 2007). (2) The second possibility can also be excluded. Electron microscopic examination demonstrated that there is no significant difference in the ischaemic signs between the non-treated and OPC-31260-treated

rats after carotid ligation. (3) We have no direct evidence relating to the effect of OPC-31260 on the cerebral capillary permeability or to a possible role of a vascular AVPR (V1) antagonist effect of OPC-31260 (Yamamura et al., 1993). (4) Most of the findings support the fourth possibility, i.e. that the renal tubular effect of OPC-31260 is the most important action in the prevention of the cerebral oedema induced by carotid ligation. Many data show that OPC-31260 antagonizes the binding of AVP to AVPR (V2) in rat kidney plasma membranes in vitro (Ishikawa et al., 1992; Yamamura et al., 1992, 1993), inducing a long-lasting and significant diuresis in vivo (Yamamura et al., 1992, 1993) by blocking the antidiuretic action of both endogenous and exogenous AVP in conscious rats (Tsuboi et al., 1994a). However, the diuretic effect of OPC-31260 is quite different from the actions of other traditional diuretic agents, such as furosemide, hydrochlorothiazide and spironolactone. The diuretic effects of these traditional diuretic drugs are closely associated with the urine Na^+ excretion, whereas OPC-31260 selectively increases the water excretion rather than that of Na^+ excretion (Yamamura et al., 1992, 1993). Grove et al. (1995) reported that infusion of the AVPR (V2) blocker OPC-31260 increased the diuresis 15-fold and tended to halve the Na^+ excretion. This selective aquaretic effect of OPC-31260 is rather advantageous with a view to the treatment of hyponatraemic cerebral oedema (Fujisawa et al., 1993).

Our findings and the above-mentioned experimental data lead us to conclude that the renal tubule-selective diuretic effect of OPC-31260 is the most important factor in the reduction of cerebral oedema following bilateral carotid ligation. Our observations might suggest a new and effective approach to the treatment of cerebral oedema following general cerebral hypoxia in humans.

Abbreviations

AVP	arginine vasopressin
AVPR (V1)	arginine vasopressin V1 receptor
AVPR (V2)	arginine vasopressin V2 receptor
RIA	radioimmunoassay

Acknowledgements

The authors are extremely grateful to Otsuka Pharmaceutical Factory for providing the OPC-31260. This study was supported by grants from OTKA (Nos. T/11 29093, T/15 42853 and T/16 046654).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 40

Characterization of a novel and selective V_{1B} receptor antagonist

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Abstract: It has been argued that hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is a major biological abnormality in patients suffering from psychiatric conditions such as major depression. Both arginine vasopressin (AVP) and corticotrophin releasing factor (CRF) are responsible for stimulating the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. CRF is thought to be the predominant secretagogue under normal conditions but AVP may play a more important role in situations of aberrant/chronic stress. Studies in patients suffering from melancholic depression indicate a hyper-responsiveness to agonism at the vasopressin receptor type 1B (V_{1B}); patients display a heightened ACTH release after challenge with the mixed V_{1B}/V_2 (vasopressin receptor type 2) agonist desmopressin in comparison to control subjects. A V_{1B} antagonist has been developed which has significant selectivity for the human V_{1B} receptor over the other members of the vasopressin receptor sub-family. The compound acts as an effective antagonist at both the human recombinant receptor (stably expressed in Chinese hamster ovary (CHO) cells) and the native rat V_{1B} receptor (using isolated anterior pituitary cells), blocking the induction of luciferase and the release of ACTH, respectively. In vivo the compound can block the release of ACTH after challenge with a variety of V_{1B} agonists. It can also attenuate the ACTH response to acute stressors in rats. Interestingly, this compound does not modulate the activity of the HPA axis under normal basal conditions.

Keywords: vasopressin; depression; HPA; ACTH; V_{1B} receptor; antagonist

Introduction

Psychiatric illnesses continue to place a major burden upon society both from an economic point of view and from the individual patient's perspective (Kessler et al., 2005). The development of front-line treatments for depression has focused almost solely upon modulation of monoamine levels in the central nervous system using first tricyclic anti-depressants (TCAs), then selective serotonin re-uptake inhibitors (SSRIs) and more recently serotonin and noradrenalin re-uptake inhibitors (SNRIs) (for a recent review of current treatments, see Nemeroff, 2007). However, these treatments still have a number of deficits including the lack of efficacy in a substantial percentage of

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DOI: 10.1016/S0079-6123(08)00440-8

patients, length of time for onset of action and unwanted side-effects such as sexual dysfunction (Fava, 2003).

One of the most commonly described biological abnormalities associated with depression is a dysfunction in the hypothalamic-pituitary-adrenal (HPA) axis. It has been well documented that a significant percentage of patients suffering from depression demonstrate elevated levels of free cortisol (Carroll et al., 1976). Furthermore, a number of studies have indicated that these patients are resistant to the HPA feedback mechanism, which is mediated by the glucocorticoid receptor (GR). Using the dexamethasone (DEX) suppression test (DST), it was found that whereas administration of DEX (a synthetic GR agonist) at night can suppress morning plasma cortisol levels in normal subjects, a large percentage of depressed patients fail to show this response, indicating an impaired negative feedback control (Carroll et al., 1981). More recently a refined version of the DST using corticotrophin releasing factor (CRF), the DEX/CRF test, has been developed. In healthy volunteers, DEX can suppress the subsequent cortisol response to CRF, whereas in depressed patients there is an enhanced response to CRF (Heuser et al., 1994). Studies have indicated that the response to the DEX/CRF test becomes normalized as patients respond to treatment and that those patients who maintain an aberrant response to the test are more likely to subsequently relapse (Holsboer-Trachsler et al., 1991).

A number of approaches to the treatment of depression by attempting to normalize the overactive HPA axis have been explored. These include the use of GR antagonists, cortisol synthesis inhibitors and CRF receptor type 1 (CRF_1) antagonists (for a recent review, see Norman and Burrows, 2007). More recently there has been growing interest in the vasopressinergic system as a means of targeting the HPA axis dysfunction.

The role of vasopressin in affective disorders

Arginine vasopressin (AVP) is a nonapeptide which acts upon a series of closely related receptors (for a review, see Thibonnier et al., 2002). AVP released from the parvocellular cells of the hypothalamus acts upon the anterior pituitary to stimulate the release of adrenocorticotropic hormone (ACTH) via the vasopressin receptor type 1B (V_{1B}) receptor. ACTH subsequently induces the release of cortisol (corticosterone in rodents) from the adrenal glands. It is believed that under normal basal conditions CRF is the major secretagogue for ACTH, whereas under situations of chronic or prolonged stress AVP gains more prominence in the control of ACTH release (Scott and Dinan, 1998). This has important implications for the feedback control on the HPA axis because whilst CRF and CRF_1 levels are negatively regulated by GR activation (Ochedalski et al., 1998), AVP signalling via V_{1B} has been reported to be enhanced by glucocorticoids (Rabadan-Diehl and Aguilera, 1998). Indeed there is increasing clinical evidence to support this hypothesis; for example post-mortem studies have demonstrated an increase in the number of AVP-expressing neurons in the paraventricular nucleus (PVN) of the hypothalamus (Purba et al., 1996). Dinan and colleagues have also carried out a number of studies probing the role of V_{1B} in depressed patients. In an initial report it was found that in comparison to healthy volunteers, depressed patients demonstrated a reduced ACTH response to a CRF challenge. However, when CRF was combined with desmopressin (dDAVP), a vasopressin analogue which activates the V_{1B} and V_2 receptors, a similar response was observed between both groups, indicating a shift in control of ACTH release towards control by AVP in depressed patients (Dinan et al., 1999). In a separate cohort of patients Dinan et al. (2004) found that infusion of dDAVP caused an enhanced release of both ACTH and cortisol in depressed patients compared to age-matched control subjects.

Development of V_{1B} antagonists

The evidence for a role of AVP/V_{1B} in depressed patients has led to an interest in the development of selective V_{1B} antagonists for the treatment of depression. The most advanced compound thus far identified, SSR149415, has been shown to be efficacious in a variety of behavioural paradigms. It demonstrates activity in the rat forced swim test, mouse tail suspension test and mouse model

of chronic mild stress (Griebel et al., 2005), tests in which monoaminergic anti-depressants have demonstrated efficacy. More recently, chronic treatment with the compound has been reported to reduce hyperemotionality in a mouse olfactory bulbectomy model (Iijima and Chaki, 2007). SSR149415 has also been reported to be efficacious in anxiety tests including the elevated plus-maze and a number of conflict paradigms (Griebel et al., 2005). SSR149415 is currently undergoing clinical trials to determine efficacy in both depression and anxiety. However, it has been recently reported that besides antagonizing the V_{1B} receptor the compound also acts as an antagonist at the closely related oxytocin (OT) receptor (Griffante et al., 2005).

Table 1. Binding affinities for the Organon V_{1b} antagonist at the human vasopressin sub-family of receptors

Receptor	p*K*i ± SEM
V_{1B}	8.4 ± 0.1
V_{1A}	<5
V_2	<5
OT	<5

Note: CHO cells (V_{1B} and OT) or membranes prepared from CHO cells (V_{1A} and V_2) expressing the human versions of the receptors were incubated with tritiated ligand ([^{3}H] AVP for V_{1A}, V_{1B} and V_2; [^{3}H] oxytocin for OT) and increasing concentrations of the compound. Non-specific binding was determined in the presence of 10 μM lysine vasopressin for V_{1A}, V_{1B} and V_2; 10 μM oxytocin for OT. The concentration of compound was plotted against specific binding and data were analysed by non-linear regression to calculate affinities of the compound for each receptor. Each determination was carried out in triplicate on at least three separate occasions. p*K*i, negative logarithm of the inhibition constant; SEM, standard error of the mean.

In vitro characterization of the Organon V_{1b} antagonist

A high-throughput screening approach was carried out by Organon in collaboration with Pharmacopeia, Inc., using a cell line stably expressing the human V_{1B} receptor to identify an initial compound with efficacy at the V_{1B} receptor, and further rounds of optimization yielded the compound. Radio-ligand binding experiments demonstrated that the compound had high affinity for the human V_{1B} receptor (approximately 4 nM) and its selectivity against other members of the vasopressin/OT receptor sub-family was found to be greater than 1000-fold (Table 1). Screening at a panel of other receptors and ion-channels indicated that the compound did not demonstrate any significant affinity (>50% binding) at a concentration of 2.5 μM.

Next the degree of functional antagonism was determined using a reporter cell line stably expressing the human V_{1B} receptor as well as a luciferase reporter gene linked to a cyclic adenosine monophosphate (cAMP) response element. Cells were stimulated with 250 nM AVP in the presence of increasing levels of the compound. The compound was able to completely block the activity of AVP at the receptor with a calculated median inhibition concentration (concentration that reduces the effect by 50%) (IC_{50}) of approximately 50 nM. Having demonstrated that it could act as an antagonist against the recombinantly expressed human receptor the next step was to determine the compound's activity at the native rat receptor. Anterior pituitary cells were prepared from female Sprague-Dawley rats. Treatment of the cells with AVP (3 nM) induced a robust release of ACTH. As can be seen in Fig. 1, the compound was able to dose-dependently block the release of ACTH ($IC_{50} = 20$ nM) when the cells were co-treated with AVP and antagonist.

In vivo characterization of the Organon V_{1b} antagonist

Blockade of V_{1B} agonist-induced HPA stimulation

The ability of the compound to antagonize the V_{1B} receptor in vivo was demonstrated by measuring ACTH release in rats following administration of CRF and dDAVP. Male adult Sprague-Dawley rats were implanted with jugular vein catheters and after a 2-day recovery period, CRF (0.3 μg/kg, intravenous (i.v.)) was administered followed by dDAVP (0.5 mg/kg, i.v.) 20 min later. After a further 10 min a blood sample was collected from each animal and plasma ACTH concentrations were measured by ELISA. As shown in Fig. 2, oral treatment with the compound two hours prior to the challenge caused a dose-related reduction in

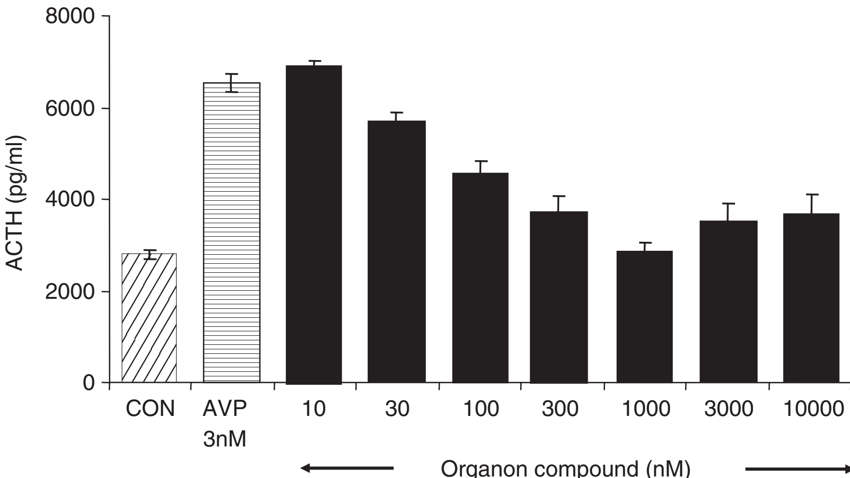

Fig. 1. The compound is able to functionally antagonize the native rat V_{1B} receptor. Isolated rat anterior pituitary cells were incubated with 3 nM AVP for 2 h in the presence of increasing concentrations of the compound. ACTH release was determined by ELISA (IDS, UK). A pIC_{50} was determined by plotting the percent antagonism against the concentration of compound ($pIC_{50} = 7.7 \pm 0.2$). Each treatment was carried out in quadruplicate and the whole experiment was repeated on at least three separate occasions. The figure is a representative result from one experiment.

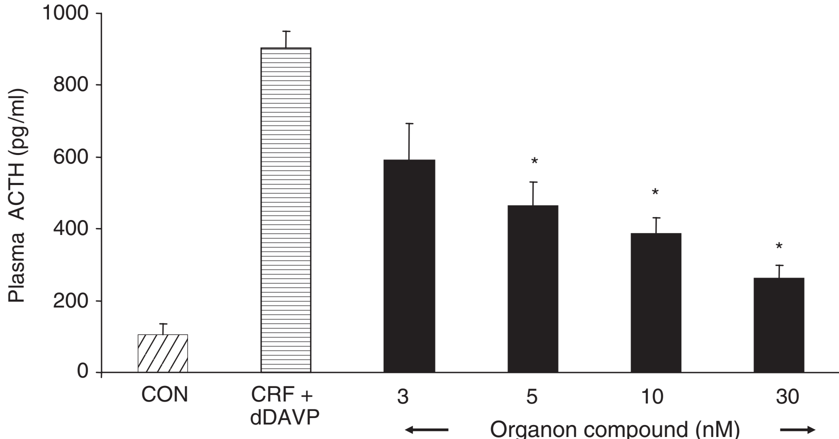

Fig. 2. The compound can antagonize the V_{1B} receptor in vivo. Male Sprague-Dawley rats (7–8 per group) were treated with the compound (3–30 mg/kg, p.o.) and then challenged with CRF (0.3 µg/kg, i.v.) and dDAVP (0.5 mg/kg, i.v.). The graph represents the mean + SEM (standard error of the mean) plasma ACTH levels. Statistical analysis was carried out on log-transformed data using a one-way ANOVA followed by Tukey's post hoc analysis. $^{*}p < 0.05$ versus group treated with CRF and dDAVP alone.

ACTH release, with a minimal effective dose of 5 mg/kg, per os (p.o.).

The in vivo efficacy of the compound was further confirmed using a selective agonist for the V_{1B} receptor, [1-deamino-4-cyclohexylalanine] arginine vasopressin (d[Cha^4]AVP), combined with CRF (Derick et al., 2002). A preliminary experiment was performed to investigate the effects of CRF and d[Cha^4]AVP, alone and in combination, on ACTH and corticosterone release. Using male Sprague-Dawley rats implanted with jugular vein catheters, CRF (0.3 µg/kg, i.v.) or vehicle was administered followed by d[Cha^4]AVP (5 or 25 µg/kg, i.v.) or vehicle 20 min later. Blood samples were

collected 10 and 30 min post-administration of d[Cha[4]]AVP for the measurement of plasma ACTH and corticosterone, respectively. Both peptides caused a significant increase in plasma ACTH levels when administered alone (see Fig. 3a). However, when administered together the resulting increase in ACTH concentrations demonstrated that the peptides were acting in a synergistic rather than additive manner. In contrast to the effect on ACTH release, d[Cha[4]]AVP alone caused a substantial increase in plasma corticosterone concentrations and the addition of CRF appeared to only have an additive effect on corticosterone release (see Fig. 3b). As displayed in Fig. 4a and b, oral treatment with the compound (30 mg/kg) effectively antagonized the release of both ACTH and corticosterone following administration of CRF and d[Cha[4]]AVP. Since the synergistic action of CRF and d[Cha[4]]AVP in stimulating the HPA axis is proposed to be mediated by the V_{1B} receptor, these results confirm that the compound can effectively antagonize the V_{1B} receptor in vivo.

In addition to its ability to block the effects of CRF combined with dDAVP or d[Cha[4]]AVP,

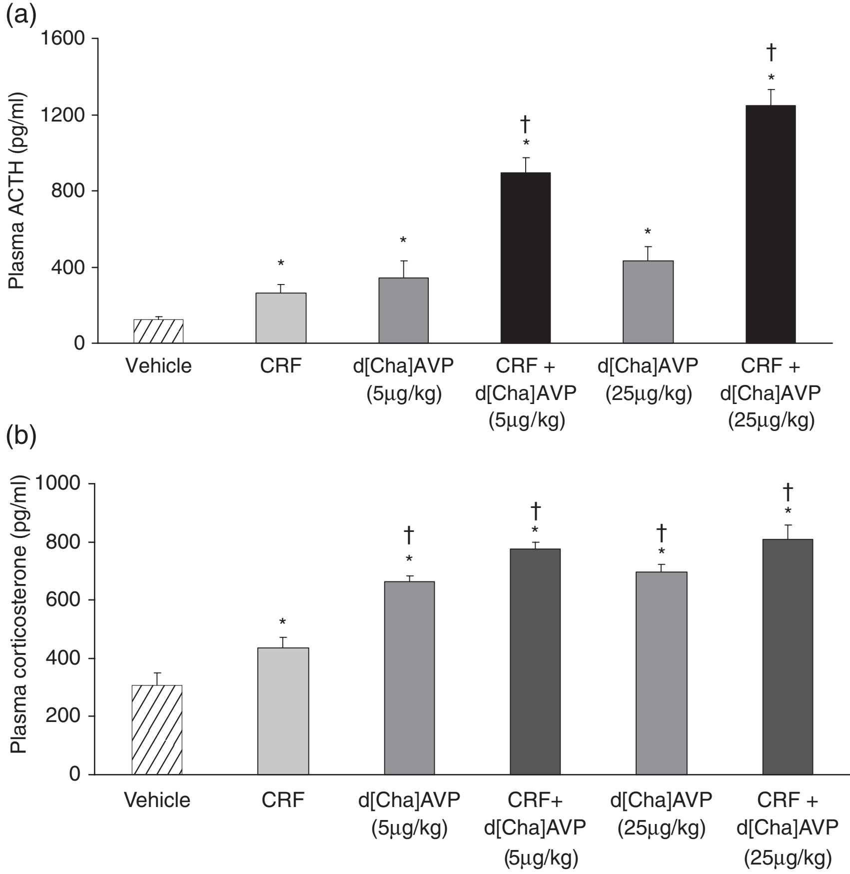

Fig. 3. (a) CRF and d[Cha[4]]AVP act synergistically to increase plasma ACTH concentrations. Male Sprague-Dawley rats (7–9 per group) were treated with CRF (0.3 µg/kg, i.v.), d[Cha[4]]AVP (at 5 or 25 µg/kg, i.v.), alone or in combination. The graph represents the mean + SEM plasma ACTH levels. Statistical analysis was carried out on log-transformed data using a one-way ANOVA followed by Tukey's post hoc analysis. $^{*}p<0.05$ versus vehicle-treated group; $^{\dagger}p<0.05$ versus CRF-treated group. (b) CRF and d[Cha[4]]AVP increase plasma corticosterone concentrations. Male Sprague-Dawley rats (7–9 per group) were treated with CRF (0.3 µg/kg, i.v.), d[Cha[4]]AVP (at 5 or 25 µg/kg, i.v.), alone or in combination. The graph represents the mean + SEM plasma corticosterone levels. Statistical analysis was carried out on log-transformed data using a one-way ANOVA followed by Tukey's post hoc analysis. $^{*}p<0.05$ versus vehicle-treated group; $^{\dagger}p<0.05$ versus CRF-treated group.

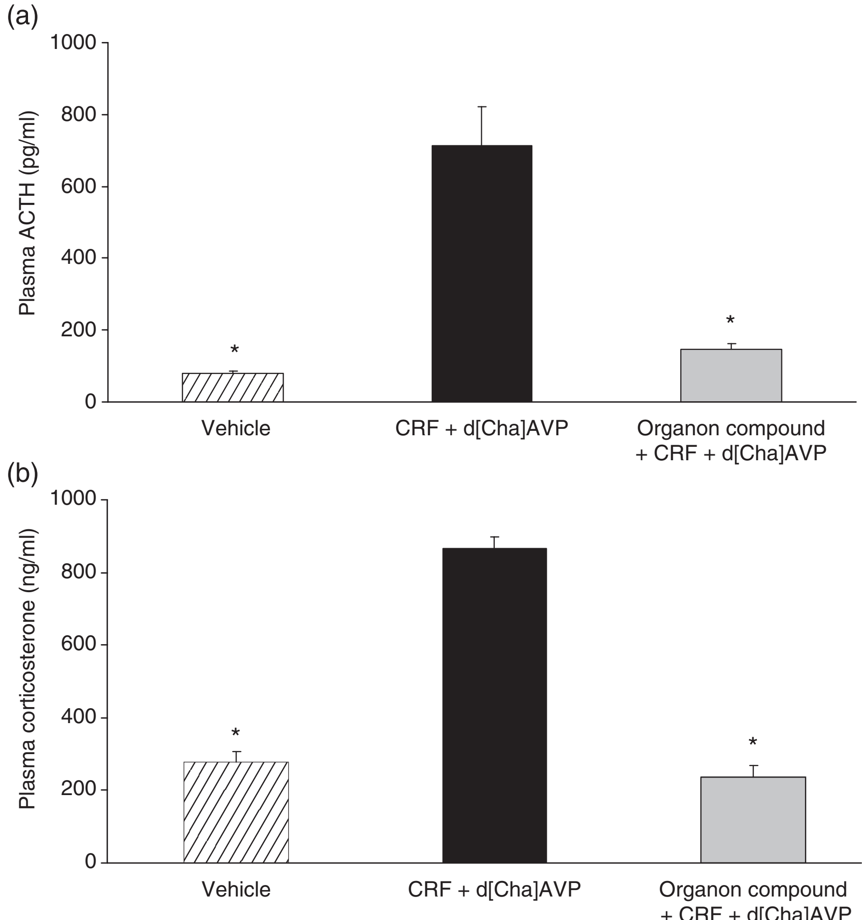

Fig. 4. (a) The compound blocks CRF and d[Cha^4]AVP-induced plasma ACTH release. Male Sprague-Dawley rats (8 per group) were treated with the compound (30 mg/kg, p.o.) and then challenged with CRF (0.3 μg/kg, i.v.) and d[Cha^4]AVP (25 μg/kg, i.v.). The graph represents the mean + SEM plasma ACTH levels. Statistical analysis was carried out on log-transformed data using a one-way ANOVA followed by Dunnett's post hoc analysis. $^{*}p<0.05$ versus group treated with CRF and d[Cha^4]AVP alone. (b) the compound blocks CRF and d[Cha^4]AVP-induced plasma corticosterone release. Male Sprague-Dawley rats (8 per group) were treated with the compound (3–30 mg/kg, p.o.) and then challenged with CRF (0.3 μg/kg, i.v.) and d[Cha^4]AVP (25 μg/kg, i.v.). The graph represents the mean + SEM plasma corticosterone levels. Statistical analysis was carried out on log-transformed data using a one-way ANOVA followed by Dunnett's post hoc analysis. $^{*}p<0.05$ versus group treated with CRF and d[Cha^4]AVP alone.

a recent study by Spiga et al. (2007) reported that the compound (30 mg/kg, subcutaneous (s.c.)) could partially antagonize the effect of AVP on ACTH secretion. Interestingly, the release of corticosterone by AVP was attenuated to a lesser extent than that observed for ACTH. The decreased magnitude of effect in blocking the corticosterone release may be a consequence of the failure to completely block ACTH release, which, albeit at low levels, could be sufficient to trigger the subsequent corticosterone release.

It was of interest to assess whether the compound would demonstrate efficacy following chronic treatment. Rats were treated with the compound for 14 days at 2.7 mg/kg/day via a subcutaneous minipump and then administered CRF and dDAVP i.v. by a jugular vein catheter (as described above). Blood samples were taken prior to the challenge and again 10 and 30 min post-administration of dDAVP

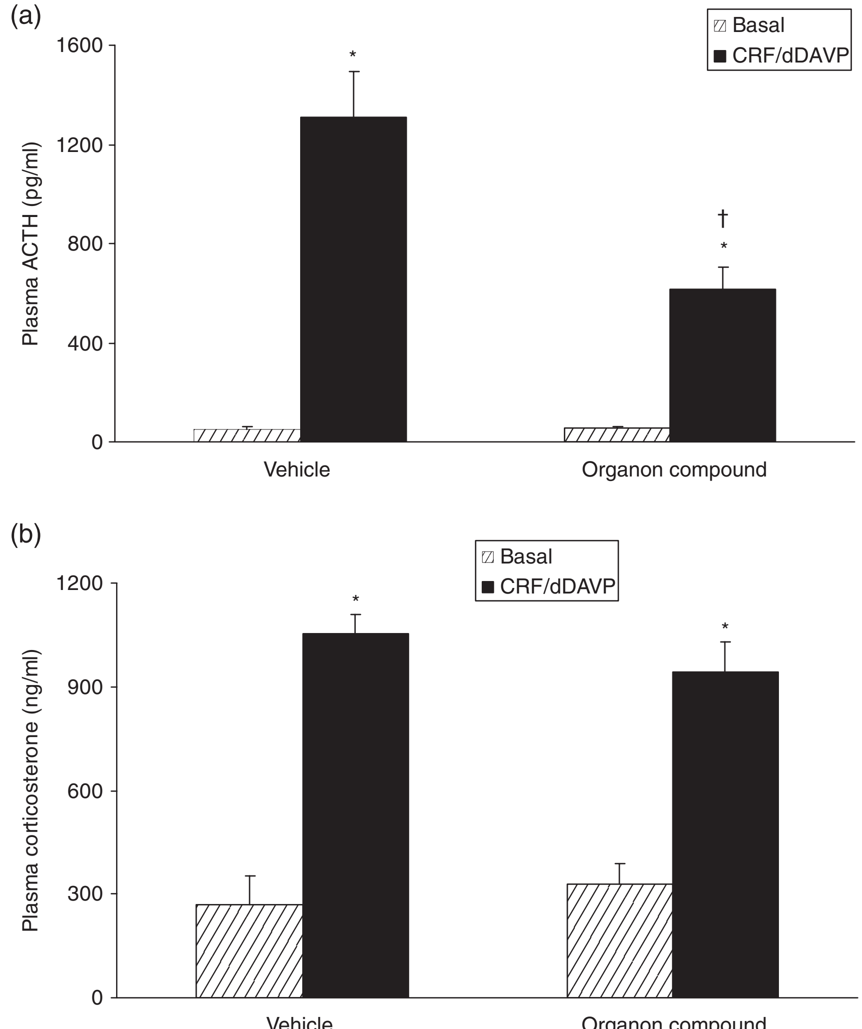

Fig. 5. (a) The compound attenuates CRF and dDAVP-induced ACTH release following chronic infusion via subcutaneous minipump. Male Sprague-Dawley rats (7–9 per group) received the compound (2.7 mg/kg/day) over 14 days by a subcutaneous minipump. They were then challenged with CRF (0.3 μg/kg, i.v.) and dDAVP (0.5 mg/kg, i.v.). The graph represents the mean + SEM plasma ACTH levels before (basal) and after the CRF and dDAVP challenge. Statistical analysis on log-transformed data using a two-way ANOVA (General Linear Model) revealed no significant effect of treatment (vehicle or Organon compound), a main effect of sample (basal or following CRF/dDAVP; $^{*}p<0.001$) and an interaction between treatment and sample ($^{\dagger}p = 0.01$). This interaction demonstrated that the drug treatment significantly attenuated the response to the CRF and dDAVP challenge. (b) the compound does not attenuate CRF and dDAVP-induced corticosterone release following chronic infusion via subcutaneous minipump. Male Sprague-Dawley rats (7–9 per group) received the compound (2.7 mg/kg/day) over 14 days by a subcutaneous minipump. They were then challenged with CRF (0.3 μg/kg, i.v.) and dDAVP (0.5 mg/kg, i.v.). The graph represents the mean + SEM plasma corticosterone levels before (basal) and after the CRF and dDAVP challenge. Statistical analysis on log-transformed data using a two-way ANOVA (general linear model) revealed no significant effect of treatment (vehicle or Organon compound) or sample (basal or following CRF/dDAVP; $^{*}p<0.001$).

for the measurement of plasma ACTH and corticosterone, respectively. Chronic treatment with the compound clearly reduced ACTH release (see Fig. 5a), demonstrating that the compound was effective as a V_{1B} antagonist following chronic treatment. Corticosterone release (Fig. 5b), on the other hand, was not reduced, which may be due to the only partial attenuation of the ACTH response.

Effects on HPA axis function

In a recent study, Spiga et al. (2007) investigated whether the compound could modulate HPA axis function under normal basal conditions. Using an automated blood sampling system, diurnal pulsatile patterns of corticosterone secretion were recorded in rats following treatment with the compound, injected acutely during the diurnal peak of the hormone secretion. The compound did not alter either the frequency or amplitude of corticosterone secretory pulses. In a separate experiment, both plasma ACTH and corticosterone levels were measured for 3 h following administration of the compound at the peak phase and no effect of the compound was observed. These data suggest that the V_{1B} receptor is not involved in basal regulation of the HPA axis. Spiga et al. (2007) also explored the effects of the compound on HPA responses to various acute stressors in rats. Plasma ACTH and corticosterone levels were measured following an acute immunological stressor (i.v. injection of lipopolysaccharide) and after a 30 min restraint stress. The compound pre-treatment partially blocked the ACTH responses to both types of acute stressor, whereas the corticosterone response remained unaltered.

Conclusion

The compound is a highly selective and potent V_{1B} receptor antagonist. It demonstrates significant binding selectivity for the human V_{1B} receptor over other members of the vasopressin receptor sub-family, as well as efficacy as an antagonist both in vitro (at the human recombinant receptor and native rat V_{1B} receptor) and in vivo, blocking ACTH release in response to challenges with various V_{1B} agonists in the rat.

The effects of the compound on HPA axis function were studied under normal basal and stressful conditions. The compound had no effect on basal circulating ACTH or corticosterone levels but it did reduce ACTH release in response to acute stressors. Plasma corticosterone levels were not reduced to the same extent; this may be due to an amplification of the corticosterone signal which only requires a sub-maximal level of ACTH to elicit a corticosterone response. Importantly, these data provide evidence that the HPA system can maintain an intact appropriate response to stressful situations in the presence of a V_{1B} antagonist. The absence of any effects on the diurnal corticosterone release in naïve animals also confirms that blockade of the V_{1B} receptor should not result in potentially harmful endocrine effects.

In summary, this compound has been shown to act at the V_{1B} receptor without disturbing the integrity of the HPA axis. Thus, the Organon V_{1b} antagonist could provide a novel approach for the treatment of anxiety and depression.

Abbreviations

ACTH	adrenocorticotropic hormone
AVP	arginine vasopressin
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CRF	corticotrophin releasing factor
CRF_1	CRF receptor type 1
d[Cha^4]AVP	[1-deamino-4-cyclohexylalanine] arginine vasopressin
dDAVP	desmopressin
DEX	dexamethasone
DST	dexamethasone suppression test
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal
i.v.	intravenous
IC_{50}	median inhibition concentration (concentration that reduces the effect by 50%)
OT	oxytocin
p.o.	per os
p*K*i	negative logarithm of the inhibition constant
PVN	paraventricular nucleus

s.c.	subcutaneous
SEM	standard error of the mean
SNRI	serotonin and noradrenalin re-uptake inhibitor
SSRI	selective serotonin re-uptake inhibitor
TCA	tricyclic anti-depressant
V_{1A}	vasopressin receptor type 1A
V_{1B}	vasopressin receptor type 1B
V_2	vasopressin receptor type 2

Acknowledgements

We thank our colleagues at Pharmacopeia Inc., Dr. Jeffrey Letourneau, Dr. Christopher Riviello, Dr. Koc-Kan Ho, Dr. Jui-Hsiang Chan, Dr. Michael Ohlmeyer, Dr. Patrick Jokiel and Dr. Irina Neagu. We are very grateful for the support we have received from Dr. Susan Napier, Dr. James Baker and Dr. Stuart Best at Organon. Thanks are also due to Dr. Greti Aguilera and Dr. Maurice Manning for providing us with d[Cha^4]AVP.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 41

Molecular mechanisms of clinical concentrating and diluting disorders

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Abstract: Impaired urinary dilution leading to water retention and hyponatremia may occur in patients with cardiac failure, cirrhosis, pregnancy, hypothyroidism, glucocorticoid and mineralocorticoid deficiency. The mechanisms for these defects predominantly involve the non-osmotic stimulation of arginine vasopressin release with upregulation of aquaporin 2 water channel expression and trafficking to the apical membrane of the principal cells of the collecting duct. These perturbations are reversed by V2 vasopressin receptor antagonists. In contrast, urinary concentration defects leading to polyuria are vasopressin-resistant. They may involve several factors, such as impaired counter-current concentration secondary to downregulation of Na-K-2Cl co-transporter. Vasopressin-resistant downregulation of aquaporin 2 expression has also been described as a factor in impaired urinary concentration.

Keywords: vasopressin; water channels; hyponatremia; V2 receptor antagonists; aquaretics

Introduction

Professor Edward B. Verney from Cambridge University performed seminal experiments which demonstrated the presence of hypothalamic osmoreceptors regulating antidiuretic hormone release from the posterior pituitary (Fig. 1; Verney, 1947). An increase in central osmolality during hypertonic injections into the internal carotid artery of a water-diuresing dog, Nicky, caused an antidiuresis earlier than injection of the same solutions into an ankle vein. Hypertonic sodium chloride, sucrose and mannitol caused comparable antidiuresis while hypertonic urea, which readily passes across cell membranes, did not alter the diuresis. Du Vigneaud synthesized arginine vasopressin (AVP) which had the biological properties of the antidiuretic hormone and he was awarded the Nobel Prize in Chemistry for this discovery (Fig. 2; Du Vigneaud et al., 1954). Our laboratory then performed a series of experiments which documented the non-osmotic, baroreceptor-mediated pathway for AVP release, which occurred independent of the osmoregulation of this antidiuretic hormone (Schrier and Berl, 1972, 1974; Schrier et al., 1972; Berl et al., 1974a, b). With the development of a radioimmunoassay, the sensitivity of the osmotic and non-osmotic pathways for AVP was demonstrated. The osmotic regulation of AVP was sensitive for 1–2% changes in plasma osmolality, while an 8–10% decrease in blood pressure was necessary to activate the non-osmotic baroreceptor pathway. Once the non-osmotic pathway is activated, however, the resultant plasma AVP levels were much higher than with osmotic stimulation (Fig. 3; Dunn et al., 1973). Neurophysiological

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DOI: 10.1016/S0079-6123(08)00441-X

Fig. 1. Edward B. Verney, FRS, Professor of Pharmacology, Cambridge, England. Adapted with permission from Verney, 1947.

studies have demonstrated that the same supraoptic neuron can be activated by either osmotic or non-osmotic baroreceptor stimulation (Fig. 4; Schrier et al., 1979). When opposing stimuli occur, such as a decrease in plasma osmolality which suppresses AVP and baroreceptor activation which stimulates AVP, a lower steady-state plasma osmolality may occur. With modest hyponatremia, these competing stimuli for AVP release may account for what has been termed a "reset osmostat", since an acute water load (20 ml/kg) may further lower plasma osmolality, override the baroreceptor stimulus and lead to urinary dilution.

Water transport across bilipid plasma membranes was shown to be too rapid for diffusion alone; therefore, tissue water channels were hypothesized. A search for water channels in various tissues was unsuccessful until a haematologist, Peter C. Agre, found an abundant protein in red blood cells (Fig. 5; Agre et al., 1993). The protein was transfected into frog eggs (*Xenopus laevis* oocytes). After 3 min in a hypotonic solution, a marked expansion occurred in the transfected frog eggs but not in normal frog eggs (Fig. 6; Preston et al., 1992). The structure of this water channel, initially termed Chip 28 and then aquaporin 1, is shown in Fig. 7 (Nielsen, 2002) and was found to be present in the proximal tubule, descending limb of Henle's loop and descending vasa rectae of the kidney. Subsequent studies by Sasaki and colleagues identified aquaporin 2 (AQP2) in the principal cells of the collecting duct (Fushimi et al., 1993). AQP2 was found to be regulated both within the short term and long term by AVP. Short-term regulation involves trafficking of AQP2 from cytoplasmic vesicles to the apical membrane of collecting duct principal cells. This involves activation of the V2 vasopressin receptor, adenylyl cyclase, cyclic AMP and protein kinase A pathway with phosphorylation of AQP2 (Fig. 8; Bichet, 2006). In the presence of the osmotic driving force initiated by the Na-K-2Cl co-transporter in the water-impermeable thick ascending limb and generated by the counter-current multiplier system, water transport enters principal cells via apical AQP2 water channels and exits via AQP3 and AQP4 water channels on the basolateral surface of these collecting duct cells. The long-term regulation of AQP2 by AVP involves a cyclic AMP response element in the AQP2 promoter with resultant increase in AQP2 protein expression. The V2 vasopressin receptor has been cloned, and recently several non-peptide V2 receptor antagonists have been developed (Schrier, 2007). On the background of these advances, the molecular mechanisms involved in a variety of clinical disorders of water homeostasis have been defined.

Congenital nephrogenic diabetes insipidus

Mutations in theV2 vasopressin receptor have been found to account for 85–90% of congenital nephrogenic diabetes insipidus (Fig. 9; Sands and Bichet, 2006). The remaining 10–15% of congenital nephrogenic diabetes insipidus is associated with mutations in the AQP2 water channel gene (Fig. 10; Sands and Bichet, 2006). Acquired nephrogenic

Cys-Tyr-Phe-Glu(NH_2)-Cys-Pro-Arg-Gly-NH_2

Fig. 2. Vicent du Vigneaud, Nobel Prize in Chemistry, 1955. Adapted with permission from Du Vigneaud et al., 1954.

diabetes insipidus has been shown to be associated with a downregulation of AQP2 in a series of experimental studies from Aarhus, Denmark, including lithium toxicity, hypokalemia, hypercalcemia, ureteral obstruction, ischaemic kidney injury and chronic renal failure (Schrier, 2006).

Primary polydipsia

Excessive water drinking in humans has been found to be associated with a diminution in maximal urinary concentration (deWardener and Herxheimer, 1957). The urinary concentrating defect secondary to polydipsia has been reproduced in the rat, thus allowing a molecular analysis of the mechanisms (Cadnapaphornchai et al., 2003b). With fluid restriction in these polyuric rats, maximal urinary osmolality was significantly diminished in spite of no change in the Na-K-2Cl transporter, the initiator of the counter-current concentrating mechanism, or medullary osmolality as compared to control rats' drinking ad libitum. During fluid deprivation, this concentrating defect was associated with elevated plasma AVP concentrations, and yet diminished AQP2 expression was observed in the polyuric rats as compared to control animals (Fig. 11; Cadnapaphornchai et al., 2003b). Thus, vasopressin-resistant downregulation of AQP2, rather than medullary "washout", explained the concentrating defect of primary polydipsia.

Hypothyroidism

Advanced hypothyroidism, as occurs with myxoedema, is associated with defects in both urinary concentration and dilution. Moreover, hypothyroid patients have been shown to not suppress plasma

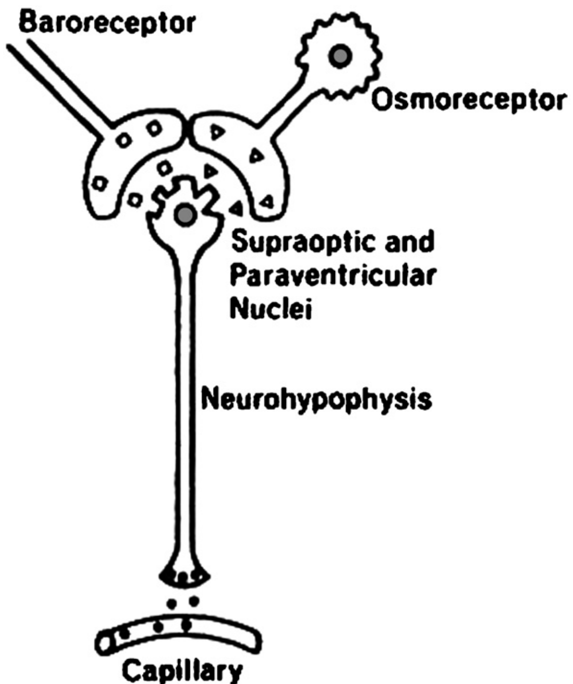

Fig. 3. Interrelationship between non-osmotic (baroreceptor) and osmotic (osmoreceptor) pathways for AVP release. Adapted with permission from Dunn et al., 1973.

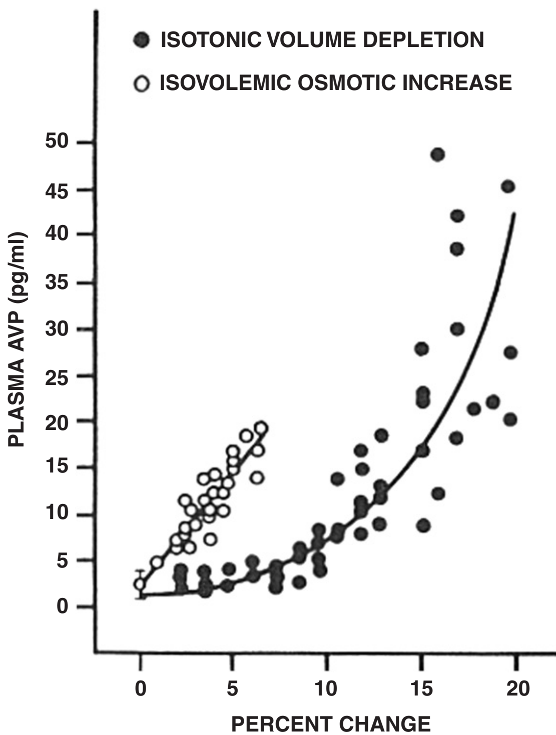

Fig. 4. Relationship of plasma AVP to percentage of change in plasma osmolality (open circles) and volume depletion (solid circles). Adapted with permission from Schrier et al., 1979.

vasopressin during an acute water load (Skowsky and Kikuchi, 1978). An experimental hypothyroid rat has been developed which demonstrates a decrease in cardiac output and slower pulse rate compared to euthyroid animals (Chen et al., 2005b). The diminished maximal urinary osmolality during fluid deprivation has been shown to exhibit a decreased expression of the Na-K-2Cl transporter and diminished medullary osmolality as well as downregulation of AQP2 and 3. This occurred in the absence of decreased plasma AVP concentration (Cadnapaphornchai et al., 2003a). The urinary dilution defect during an acute water load was profoundly diminished in the hypothyroid as compared to euthyroid animals. Moreover, the plasma vasopressin concentration and AQP2 expression were significantly increased in the hypothyroid animals (Fig. 12; Chen et al., 2005b). The impaired response to an acute water load in hypothyroid rats was totally reversed including suppression of plasma vasopressin and AQP2 with thyroxin replacement (Fig. 12). The percentage of acute water load excreted and urinary osmolality were normalized with a V2 receptor antagonist (Fig. 13; Chen et al., 2005b).

Glucocorticoid deficiency

Glucocorticoid deficiency occurs with hypopituitarism and may be associated with hyponatremia. Physiological doses of hydrocortisone have been shown to correct the hyponatremia associated with hypopituitarism (Agus and Goldberg, 1971). The hyponatremia has been shown to be mediated by the non-osmotic stimulation of vasopressin (Chen et al., 2005a). Studies have been undertaken in adrenalectomized rats replaced only with mineralocorticoid hormone, as compared to control adrenalectomized rats replaced with both glucocorticoid and mineralocorticoid hormones (Chen et al., 2005a; Wang

Fig. 5. Peter C. Agre, MD, Professor of Medicine, Duke University. Nobel Prize in Chemistry, 2003 for the discovery of water channels.

et al., 2006). Maximal urinary concentration was diminished in the glucocorticoid deficient rat in association with a significant decrease in Na-K-2Cl co-transporter, medullary osmolality, AQP1 and AQP2 and urea transporter 1 (UTA1) (Chen et al., 2005a). The response to an acute water load was profoundly impaired in association with an increase in plasma vasopressin and AQP2 expression. The administration of a V2 receptor antagonist normalized the defect in urinary dilution and the elevated AQP2 in glucocorticoid-deficient animals (Fig. 14; Wang et al., 2006).

Mineralocorticoid deficiency

In contrast to glucocorticoid deficiency, mineralocorticoid deficiency is a renal sodium wasting state, which may be associated with renal hyperkalemia and non-anion gap metabolic acidosis. In experimental mineralocorticoid deficiency, the impaired urinary concentrating defect was associated with a diminution in Na-K-2Cl co-transporter and an increase in AQP2 and AQP3 (Ohara et al., 2002). These perturbations were totally reversed by avoiding the negative sodium balance

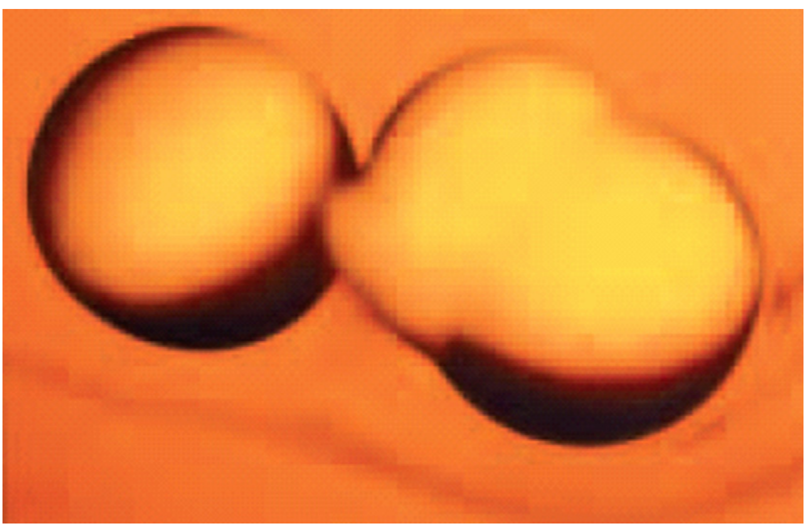

Fig. 6. Functional expression of AQP1 water channels on *Xenopus laaevis* oocytes after 3 min in hypotonic solution. Adapted with permission from Preston et al., 1992.

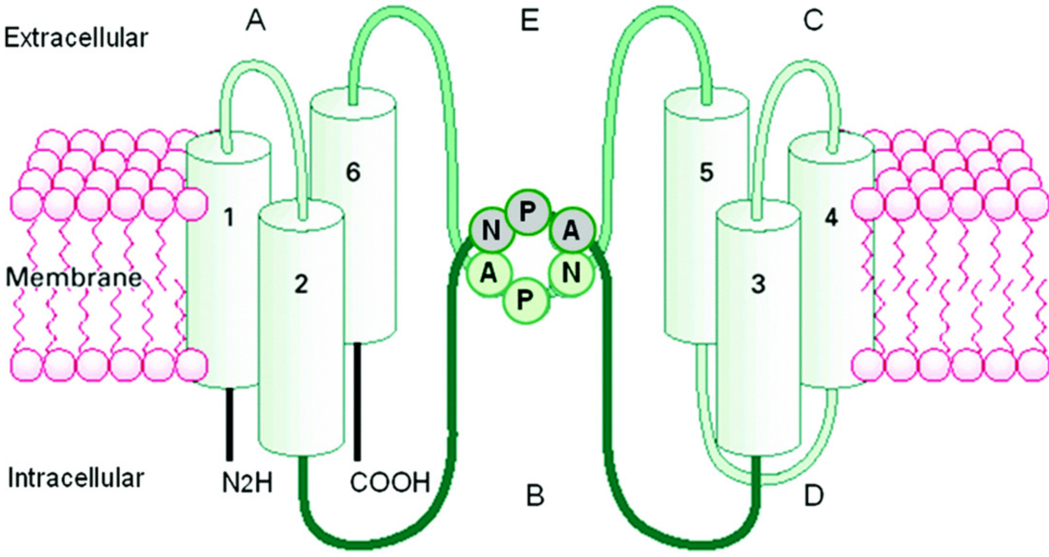

Fig. 7. Schematic representation of the antidiuretic hormone, arginine-vasopressin. Adapted with permission from Bichet, 2006. (See Color Plate 41.7 in color plate section.)

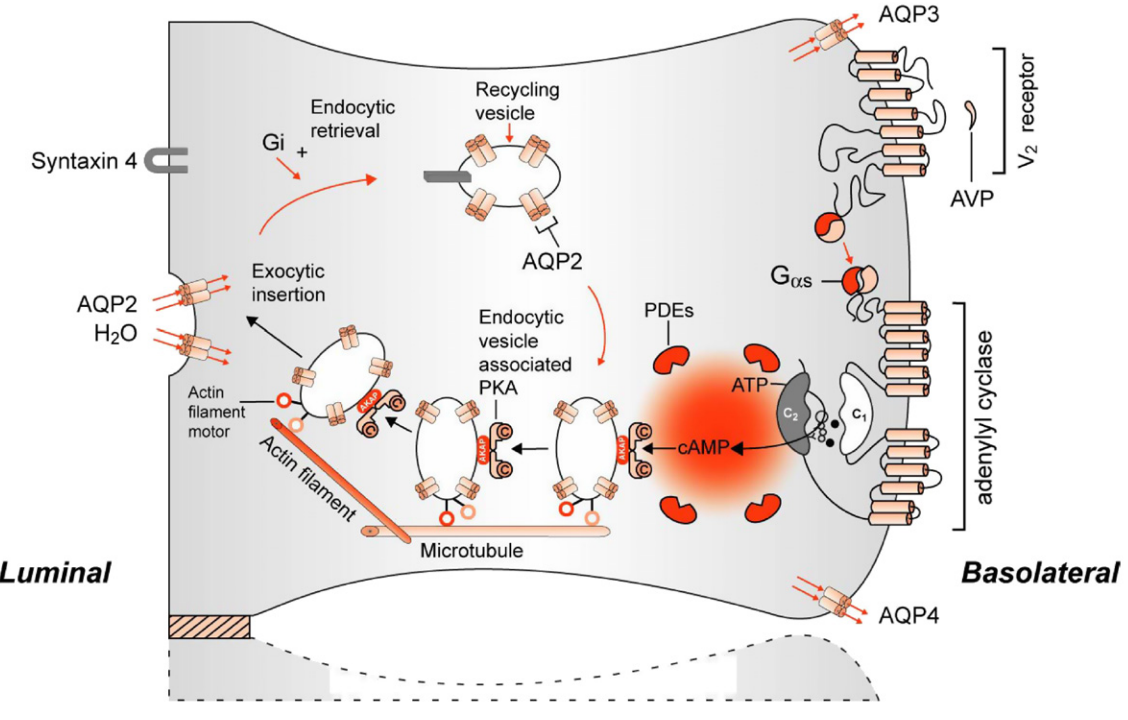

Fig. 8. Intracellular action of the antidiuretic hormone, arginine-vasopressin. Adapted with permission from Sands and Bichet, 2006. (See Color Plate 41.8 in color plate section.)

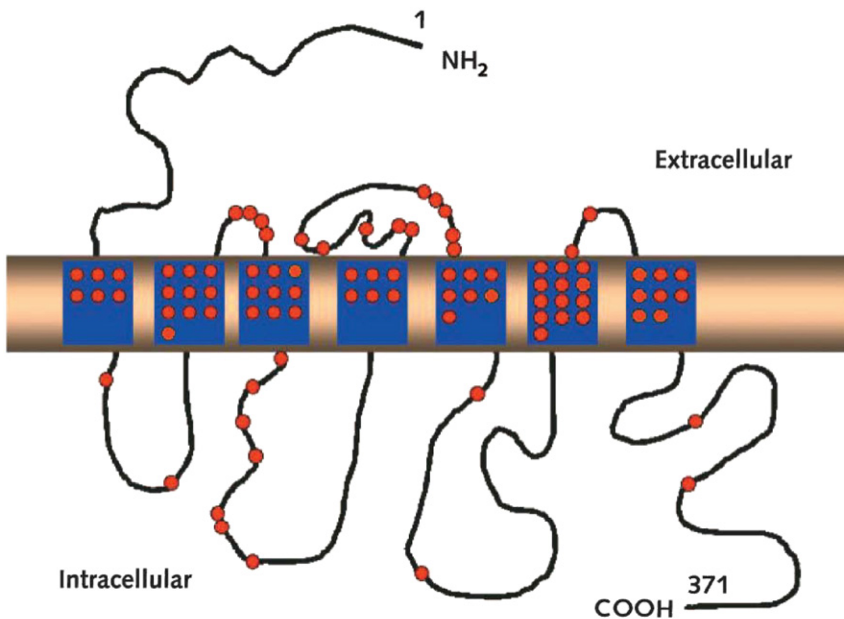

Fig. 9. Mutations in V2 receptor causing congenital nephrogenic diabetes insipidus. Adapted with permission from Sands and Bichet, 2006. (See Color Plate 41.9 in color plate section.)

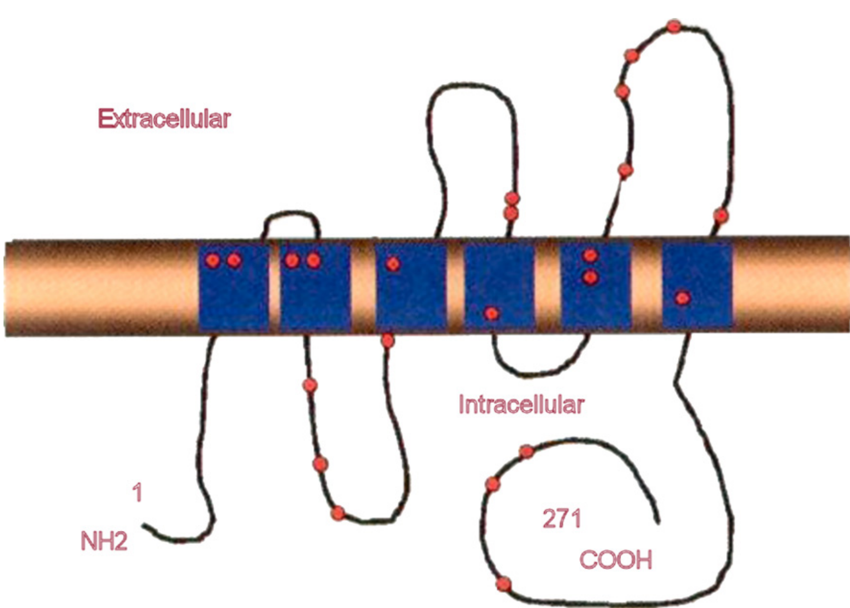

Fig. 10. Mutations in AQP2 water channel causing congenital nephrogenic diabetes insipidus. Adapted with permission from Sands and Bichet, 2006. (See Color Plate 41.10 in color plate section).

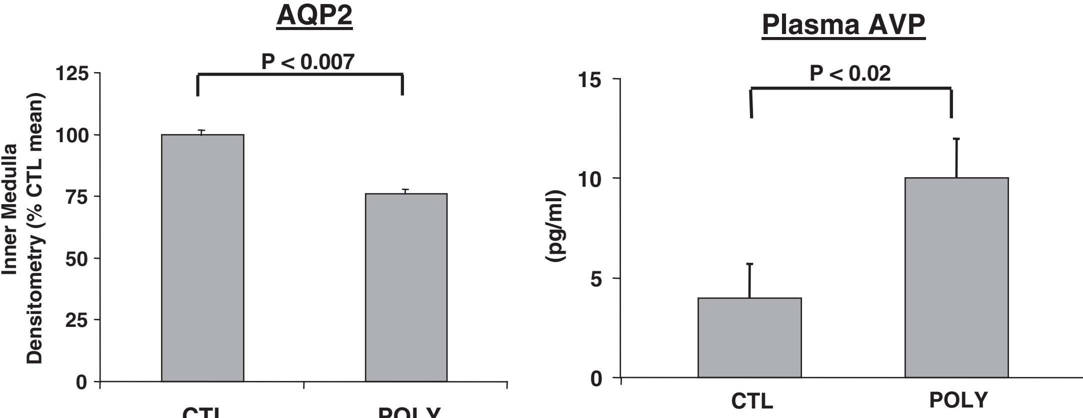

Fig. 11. Decreased AQP2 water channel expression in polydipsic rats during fluid restriction. Adapted with permission from Cadnapaphornchai et al., 2003b.

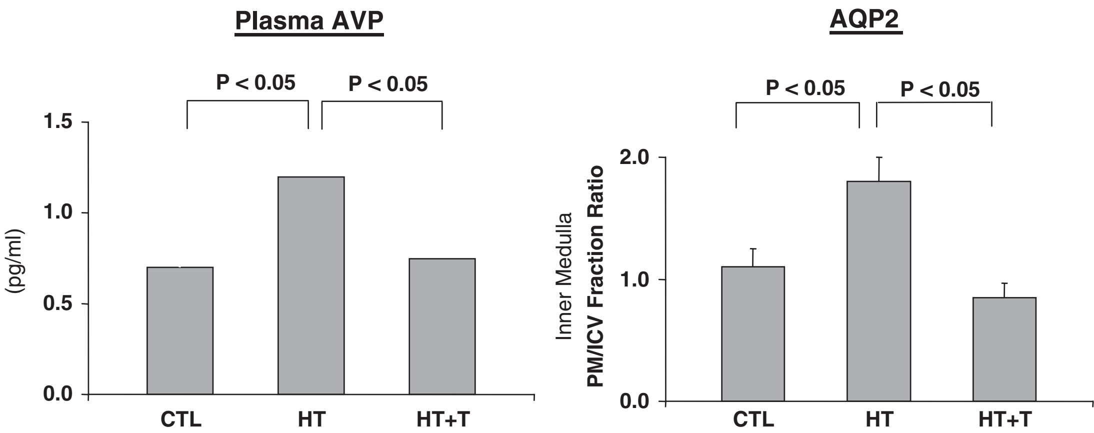

Fig. 12. Impaired water excretion in hypothyroidism: role of vasopressin-mediated AQP2. Adapted with permission from Chen et al., 2005b.

in mineralocorticoid-deficient animals with saline drinking water. The role of non-osmotic vasopressin in the hyponatremia of mineralocorticoid deficiency was supported by the improved urinary dilution with a V2 vasopressin antagonist (Ishikawa and Schrier, 1982).

Cardiac failure

Hyponatremia, independent of treatment, occurs in advanced cardiac failure and is a poor prognostic factor (Lee and Packer, 1986). Plasma vasopressin concentrations have been shown to be increased in hyponatremic heart failure patients (Szatalowicz et al., 1981). In experimental heart failure secondary to coronary ligation rats, an increase in AQP2 expression and membrane trafficking have been demonstrated (Nielsen et al., 1997; Xu et al., 1997). Administration of V2 vasopressin antagonist was shown to increase solute-free water excretion and correct hyponatremia in association with normalizing AQP2 expression and membrane trafficking (Xu et al., 1997). On this background, several V2 vasopressin antagonists have been shown to increase plasma sodium concentration in

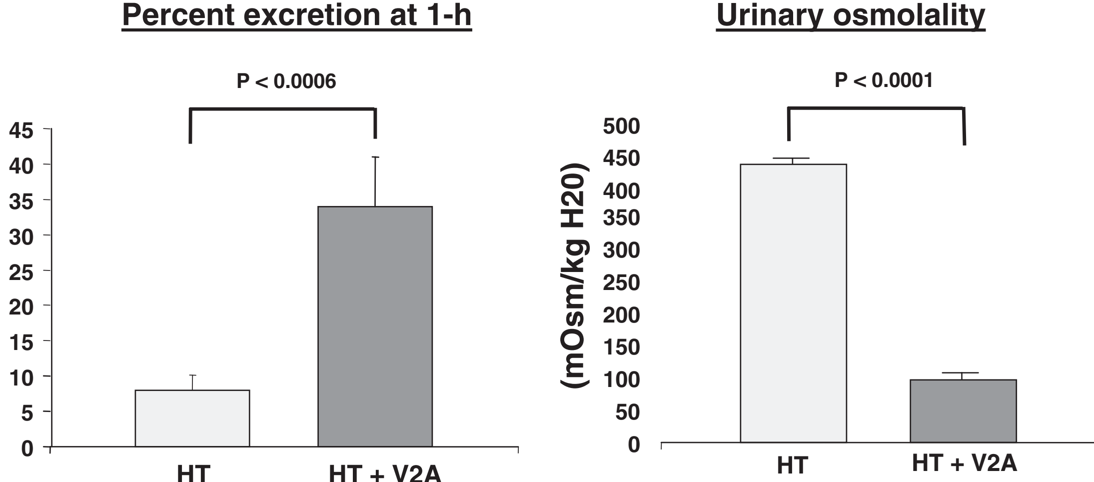

Fig. 13. Reversal of impaired water excretion during hypothyroidism (HT) with V2 receptor antagonist (V2A). Adapted with permission from Chen et al., 2005b.

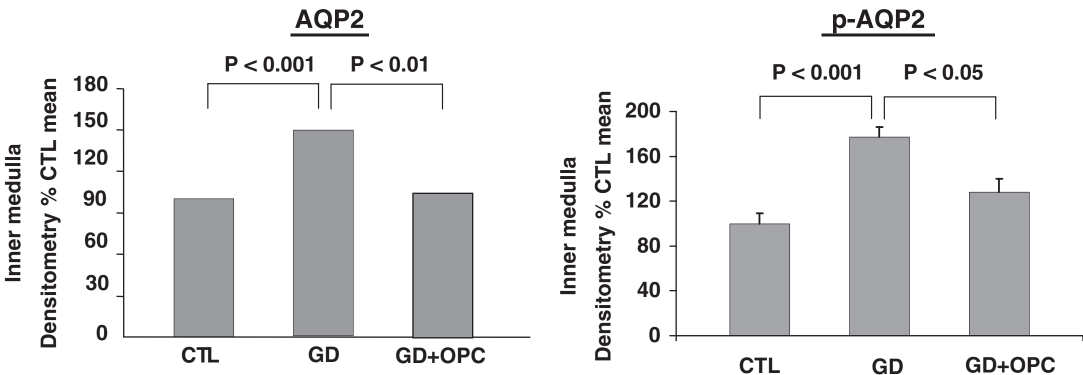

Fig. 14. Reversal of impaired water excretion in glucocorticoid deficiency with V2 receptor antagonist (OPC). Adapted with permission from Chen et al., 2005a.

hyponatremic cardiac failure patients in association with an increase in solute-free water excretion (Gheorghiade et al., 2004) and a decrease in urinary AQP2 excretion (Martin et al., 1999).

Cirrhosis

Pretreatment hyponatremia occurs in advanced cirrhosis and is a risk factor for progression to hepatorenal syndrome and mortality (Gines et al., 1993). Plasma vasopressin concentrations are not suppressed in these patients and the response to

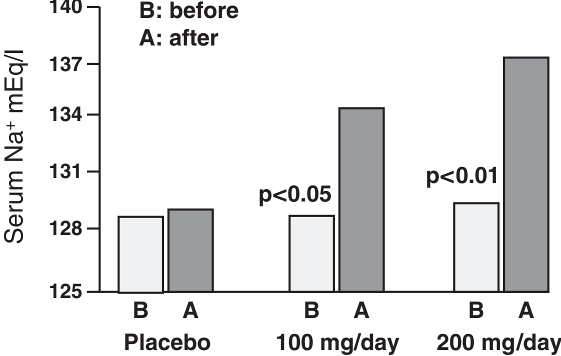

Fig. 15. Lixivaptan in hyponatremic *cirrhotic patients*. Adapted with permission from Gerbes et al., 2003.

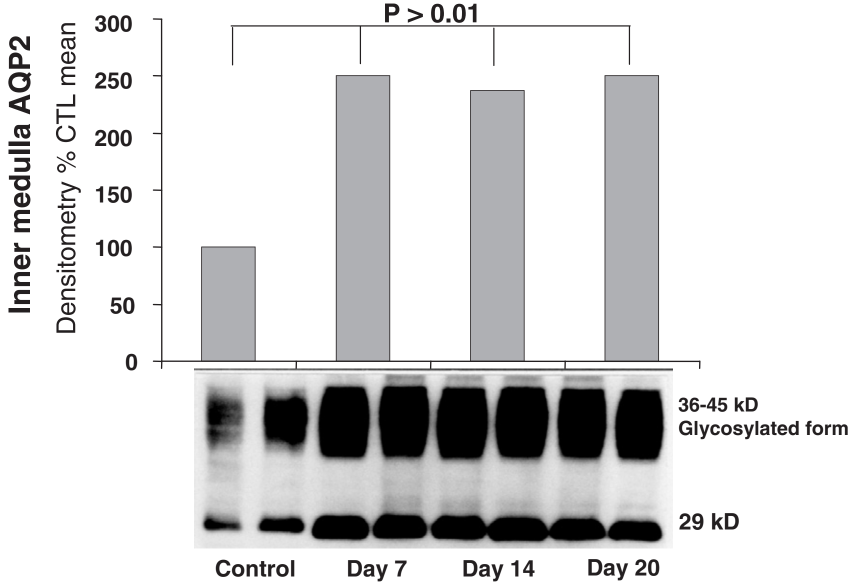

Fig. 16. Protein expression of inner medullary AQP2 in pregnancy. Adapted with permission from Ohara et al., 1998.

an acute water load is impaired (Bichet et al., 1982). This finding is compatible with non-osmotic baroreceptor-mediated vasopressin release, secondary to splanchnic arterial vasodilation and thus relative arterial underfilling. In experimental cirrhosis with intraperitoneal CC14, the impaired response to an acute water load is associated with failure to suppress AQP2 message (Asahina et al., 1995). On this background, studies in hyponatremic cirrhotic patients using different V2 vasopressin receptor antagonists have been undertaken (Gerbes et al., 2003; Schrier et al., 2006). In these studies, an increase in electrolyte-free water excretion and an increase in plasma sodium concentration have been observed (Fig. 15; Gerbes et al., 2003).

Pregnancy

Human pregnancy is associated in the first trimester with arterial vasodilation, and this arterial underfilling is associated with a decrease in plasma osmolality, failure to suppress plasma vasopressin, activation of the renin–angiotensin–aldosterone system and a secondary rise in cardiac output (Chapman et al., 1998). Pregnancy in rats mimics these hormonal and haemodynamics of human pregnancy. In rat pregnancy, a profound increase in renal medullary AQP2 occurs (Fig. 16; Ohara et al., 1998). With a V2 vasopressin receptor antagonist, a reversal of the water retention and increase in electrolyte-free water excretion. In human pregnancy, an increase in urinary AQP2 has been observed (Buemi et al., 2001).

Thus, in several hyponatremic states in humans and experimental animals has been shown to be associated with baroreceptor-mediated non-osmotic release of vasopressin in association with increased renal AQP2 expression and trafficking. These perturbations have been shown to be reversed with V2 vasopressin receptor antagonist.

Acknowledgements

This work has been supported by the NIH Vasopressin Grant at the University of Colorado. The author would like to thank Jan Darling for her editorial support.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 42

Actin-binding channels

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Abstract: Channel proteins play essential roles in physiology including ion and volume homeostasis and signal transduction and in pathophysiology of many human diseases. Recently, the importance of the actin cytoskeleton in the channel protein regulation has been highlighted. The actin cytoskeleton and its reorganization have been reported to be required for the regulation of both channel activity and its intracellular trafficking. Furthermore, there are increasing evidences showing the direct interactions of channel to actin. This review focuses on actin-binding channel proteins and the role of the actin interaction in the channel protein regulation.

Keywords: actin remodelling; membrane trafficking; aquaporin

Introduction

Channel proteins are essential for a variety of physiological and pathophysiological processes including maintenance of intra- and extracellular ionic gradients, volume homeostasis and signal transduction. There are two targets for channel regulation: channel activity and its intracellular localization. For both ways of regulation, the importance of the actin cytoskeleton was shown using actin-modulating drugs. Furthermore, recent development of molecular and proteomic approaches has revealed the direct interaction of several channels to actin itself. We previously showed that aquaporin-2 (AQP2) directly binds to actin (Noda et al., 2004b, 2005; Noda and Sasaki, 2006). To date, in addition to AQP2, other five channels have been reported to bind to actin directly: ClC2 and short isoform of ClC3 (sClC3) chloride channel, vacuolar type H^+-ATPase (V-ATPase), voltage-dependent anion channel (VDAC) and α subunit of epithelial sodium channel (ENaC). In this review, we describe current findings indicating actin-binding channels and the role of the actin interaction in the channel regulation.

ClC2 and sClC3 chloride channel

ClC2 and sClC3 (short ClC3 isoform) are hypotonic cell swelling-sensitive channels belonging to a voltage-regulated chloride channel family (Duan et al., 1997; Furukawa et al., 1998; Jentsch et al., 1999). The activation of these channels contributes to the maintenance of physiological cell volume. These channels have different biophysical properties: ClC2 exhibits inward rectification, whereas sClC3 exhibits outward rectification. However,

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DOI: 10.1016/S0079-6123(08)00442-1

actin remodelling is reported to be involved in the activation of both channels (Ahmed et al., 2000; Wang et al., 2005). Furthermore, it has been demonstrated that ClC2 N terminus and sClC3 C terminus directly bind to actin, respectively (Ahmed et al., 2000; McCloskey et al., 2007).

Jentsch and co-workers show that mutations in the N-terminus and in the the cytoplasmic loop between transmembrane spans 7 and 8 of ClC2 lead to a constitutive activation with a loss of swelling- and voltage-sensitivity and suggest that the interaction between these domains inhibits the channel opening (Gründer et al., 1992; Jordt and Jentsch, 1997). Bear and co-workers show that the N terminal domain fused to glutathione S-transferase (GST) is capable of binding actin in overlay and cosedimentation assay (Ahmed et al., 2000). Furthermore, the chloride channel activity of ClC2 in *Xenopus* oocyte is enhanced by actin-disrupting agents cytochalasin and latrunculin. Although the binding of the whole molecule of ClC2 to actin is not confirmed, these findings suggest that the intramolecular association between the N-terminus and the loop between transmembrane spans 7 and 8 of ClC2 may be stabilized through associations with the actin cytoskeleton, which results in the channel inactivation.

Yamboliev and co-workers show a strong binding between cytosolic C terminus of sClC3 fused with GST (GST-sClC3-CT) and F-actin by cosedimentation assays (McCloskey et al., 2007). Inhibition of the interaction by synthetic peptides corresponding to the binding domain leads to a reduced hypotonic activation of sClC3. The C terminus of sClC3 also contains a tandem repeat of cystathionine-β-synthase (CBS)-like domains, which have been proposed to affect multimerization and sorting of proteins, channel gating and ligand binding (Ignoul and Eggermont, 2005). It is speculated that F-actin binding to the C terminus of sClC3 may induce ordered folding of the CBS tandem and lead to the hypotonic activation of sClC3.

V-ATPase

Vacuolar type H^+-ATPase (V-ATPase) is the most versatile proton pump, highly conserved among all eukaryotic organisms, and expressed in the intracellular membrane systems and in the plasma membrane (Nishi and Forgac, 2002). V-ATPase is essential for cellular pH homeostasis and creates an electrochemical proton gradient. Acidification of organelles by V-ATPase is critical for many cellular processes including neurotransmitter uptake into synaptic vesicles (Beyenbach and Wieczorek, 2006). V-ATPase also is able to acidify the extracelllular compartment that serves a number of roles such as bone reabsorption by osteoclasts and urinary acidification (Beyenbach and Wieczorek, 2006). V-ATPase is composed of a catalytic ATP-hydrolysing V_1 complex residing on the cytoplasmic side of the membrane, and a membrane-bound proton-translocating V_0 complex. The V_1 complex contains eight different subunits, A–H, whereas the V_0 complex consists of four different subunits a and c–e (Merzendorfer et al., 2000). V-ATPase activity is down-regulated by reversible dissociation of the V_1 complex from the membrane and during this process subunit C gets lost from the V_1 complex (Kane, 1995, 2000; Sumner et al., 1995; Merzendorfer et al., 2000). However, subunit C is necessary for reassembly of the two complexes into a functional holoenzyme.

There are several reports showing the interactions of V-ATPase with actin. During osteoclast activation, V-ATPase directly binds to F-actin with high affinity and this association is correlated with V-ATPase transport from the cytoplasm to the plasma membrane (Lee et al., 1999). Because the complex with F-actin and V-ATPase also contains myosin II, Holliday and co-workers suggest that myosin II-powered contraction of microfilaments transports V-ATPase to the site of nascent ruffled membranes. The same group identifies an F-actin binding site in the amino-terminal domain of the subunit B (Holliday et al., 2000). In addition to subunit B, subunit C, which gets released during reversible dissociation of the holoenzyme, is also shown to bind to F-actin (Vitavska et al., 2003). Furthermore, Vitavska et al. (2005) demonstrate that subunit C binds not only to F-actin but also to monomeric G-actin. Subunit C has at least two actin-binding sites in its N- and C-terminal halves and is shown to cross-link actin filaments. This interaction may be involved in stabilising V-ATPase

in its assembled state because subunit C appears to bridge the V_1 with the V_0 complex (Inoue and Forgac, 2005). Recently, recombinant subunit C is shown to be phosphorylated by protein kinase A (PKA; Voss et al., 2007). Furthermore, subunit C is phosphorylated by cAMP-analogue 8-CPT-cAMP and the neurohormone serotonin in salivary glands of the blowfly *Calliphora vicina* in which V-ATPase reassembly and activity is regulated by serotonin via PKA (Voss et al., 2007). Voss et al. suggest that subunit C phosphorylation may facilitate the reassembly to an active V_1V_0 holoenzyme. In addition, subunit C interaction with actin may be regulated by its phosphorylation and be involved in the regulation of V-ATPase.

VDAC

VDAC is a channel-forming protein in the mitochondrial outer membrane responsible for metabolic flux through that membrane (Colombini, 2004). VDAC has been implicated in the initiation of the mitochondrially mediated pathway of apoptosis. Although the mechanism of action is still controversial, closure of the VDAC and the subsequent decrease in metabolic flux may cause the permeabilization of the outer membrane to relatively small proteins, leading to apoptosis (Rostovtseva et al., 2005). The gating of VDAC is influenced by a variety of proteins including Bcl-x_L (Vander Heiden et al., 2001), heat shock protein mtHSP70 (Schwarzer et al., 2002), dynein light chain (Schwarzer et al., 2002) and G-actin (Xu et al., 2001). G-actin induces the channel closure, and DNase-I, a protein that binds tightly to actin, blocks this effect (Xu et al., 2001). Surface plasmon resonance experiments show the direct binding of VDAC to G-actin (Roman et al., 2006). How this interaction regulates VDAC function remains unclear.

ENaC

Amiloride-sensitive ENaC functions in the movement of sodium across epithelial cells in a variety of tissues and is importantly involved in the control of extracellular fluid volume and blood pressure (Snyder, 2002). Several lines of evidence have indicated the involvement of actin in the regulation of ENaC. Patch-clamp experiments showed that ENaC activity in cell-attached patches from A6 cells was increased by cytochalasin D and short actin filaments, respectively (Cantiello et al., 1991). Short actin filaments increased open probability of αβγENaC reconstituted in planar lipid bilayers (Berdiev et al., 1996). Using a series of truncation and deletion mutants of αENaC reconstituted in planar lipid bilayers, Copeland et al. (2001) showed that the region between residues 631 and 644 in the carboxy terminus was important for the regulation by actin.

The involvement of actin in the intracellular trafficking of ENaC has also been reported. In mouse cortical collecting duct epithelial cells, an actin-depolymerizing agent latrunculin A inhibits ENaC exocytosis (Butterworth et al., 2005).

Mazzochi et al. (2006a, b) show the direct interaction of αENaC with actin. In MDCK cells stably expressing αβγENaC, αENaC is colocalized with cortical F-actin and F-actin is coimmunoprecipitated with αENaC. Gel overlay assays and cosedimentation assays show that F-actin binds directly and specifically to the C terminus of αENaC. The colocalization of αENaC and F-actin in the subapical cytoplasm suggests that in addition to regulating ENaC activity, the direct interaction between F-actin and ENaC may function in the intracellular trafficking of ENaC from a subapical pool to the plasma membrane.

AQP2

AQP2 is the predominant vasopressin-regulated water channel (Fushimi et al., 1993). Trafficking of AQP2 to the apical membrane and its vasopressin and PKA-dependent regulation in renal collecting ducts is critical for body water homeostasis (Noda and Sasaki, 2006). There are many reports showing the involvement of actin in the intracellular trafficking of AQP2. Forskolin and okadaic

acid stimulate AQP2 translocation by inducing a reorganization of the apical actin network, respectively (Valenti et al., 2000). Actin depolymerization is a prerequisite for cAMP-dependent translocation of AQP2 (Klussmann et al., 2001; Tamma et al., 2001). These findings indicate a role of the actin depolymerization in the vasopressin-induced translocation of AQP2 from intracellular vesicles to the cell surface. Furthermore, RhoA inhibition is required for AQP2 translocation (Tamma et al., 2003a). Stimulation of prostaglandin E3 receptors inhibits vasopressin-induced Rho inactivation, vasopressin-induced F-actin depolymerization, and vasopressin-, cAMP- and forskolin-induced AQP2 translocation (Tamma et al., 2003b). Rho activation by Bradykinin stabilizes cortical F-actin and inhibits AQP2 trafficking (Tamma et al., 2005).

Not only inhibitory effects but also facilitatory effects of F-actin assembly on AQP2 trafficking have been suggested. Actin-depolymerizing agent cytochalasin D did not enhance water permeability of the epithelium of toad urinary bladder (Franki et al., 1992). The authors speculate that F-actin has at least two hypothetical pools: one is involved in the barrier function and the other pool is involved in the transport of AQP2. Tajika et al. (2005) show that actin depolymerization caused by cytochalasin D or latrunculin B inhibits AQP2 translocation from EEA1-positive early endosomes to Rab11-positive subapical storage vesicles. Furthermore, vasopressin signalling induces myosin light chain phosphorylation which is known to enhance myosin–actin filament interaction and the formation of actin fibres (Chou et al., 2004). Recently, myosin is shown to be critical for AQP2 recycling (Nedvetsky et al., 2007).

Since both stimulatory and inhibitory effects of actin assembly are observed in AQP2 trafficking, regulation of actin remodelling may be different by time and localization during the translocation. In the process that the physiological trafficking of AQP2 occurs in vivo, the actin dynamics may change in a restricted narrow area around AQP2 molecule and may vary among different areas within the cell.

Recently, we have clarified that AQP2 directly binds to actin and SPA-1 (Noda et al., 2004a, b, 2005). SPA-1 is a GTPase-activating protein (GAP) for Rap1 and the GAP activity of SPA-1 is required for AQP2 trafficking to the apical membrane. Since Rap1 affects the assembly of F-actin (Tsukamoto et al., 1999; Pak et al., 2001; Harazaki et al., 2004; Kometani et al., 2004; Noda and Sasaki, 2006), SPA-1 binding to AQP2 may reduce the levels of Rap1GTP that trigger F-actin disassembly in a restricted area, resulting in the promotion of the AQP2 trafficking. Indeed, AQP2 trafficking to the apical membrane is impaired in the collecting duct principal cells of SPA-1 deficient mice (Noda et al., 2004a). SPA-1 deficient mice show marked bilateral hydronephrosis due to polyuria (Noda et al., 2004a; Kometani et al., 2006).

In addition to actin and SPA-1, AQP2 is shown to form a complex with 11 proteins: ionized calcium binding adapter molecule 2, myosin regulatory light chain smooth muscle isoforms 2-A and 2-B, α-tropomyosin 5b, annexin A2 and A6, scinderin, gelsolin, α-actinin 4, α-II spectrin and myosin heavy chain nonmuscle type A (Noda et al., 2005). Since these proteins have actin binding abilities, each interaction of these proteins in the complex may be dynamic and this dynamic assembly acts as a key point for the regulation of the AQP2 trafficking. To clarify the biophysical mechanisms which provide force driving AQP2 movement, we are now examining the interaction dynamics of AQP2 with these binding proteins at the resolution of single molecule during AQP2 translocation.

Concluding remarks

In recent years evidence has accumulated to demonstrate the direct binding of channel proteins to actin. However, the roles of the direct interaction in the regulation of many channels have not been confirmed. There are several reasons for the difficulty in examining the roles. For example, high concentrations of actin molecules in the cell make the microscopic colocalization assessment difficult.

Actin-modulating agents inevitably affect the overall cell architecture which makes the experiments examining the roles of actin interaction in channel regulation difficult. The roles and the regulation mechanisms will be uncovered by application of time lapse imaging with high spatiotemporal resolution and other methods that enable to assess the real-time molecular dynamics in a small area in live cells.

Acknowledgement

We thank Dr. Saburo Horikawa for useful discussions and comments.

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 43

Opposite potentiality of hypothalamic coexpressed neuropeptides, apelin and vasopressin in maintaining body-fluid homeostasis

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Abstract: This review concentrates on the characteristics and functionality of endocrine neurons in the hypothalamo-neurohypophysial system, coexpressing two peptides, vasopressin and apelin. Vasopressin is synthesized in the soma of magnocellular neurons, then packaged in granules with its respective receptors. In these neurons, apelin is generated from a larger precursor proapelin and is detected in vesicles, some of them colocalize with vasopressin, for others there is a marked segregation of apelin and vasopressin immunoreactivity along the hypothalamo-hypophyseal axons. Furthermore, apelin receptors, like V1a-type and V1b-type vasopressin receptors, are synthesized by magnocellular vasopressin neurons. In lactating rodents, apelin given intracerebroventricularly inhibited the phasic electrical activity of vasopressin neurons, reduced plasma vasopressin levels and increased aqueous diuresis, showing that apelin acts as a potent diuretic neuropeptide, counteracting vasopressin actions through inhibition of vasopressin neuron activity and vasopressin release. Moreover, in response to potent physiological stimuli known to evoke increased phasic activity of vasopressin neurons (hyper-osmolarity like during dehydration), both the soma dendrites and neurohypophysial terminals loose their dense staining quality, and vasopressin is released by (i) dendrites in the extracellular space to optimize the characteristic phasic activity necessary to a sustained release of vasopressin and (ii) by terminals in blood circulation where vasopressin then ensures its main endocrine actions at kidney level (antidiuretic effect). Conversely, apelin accumulates in these neurons rather than being released into the bloodstream and probably into the nuclei. Thus, decreases in the local supply of apelin to magnocellular vasopressin cell bodies may facilitate the expression by vasopressin neurons of an optimized phasic activity, by decreasing the inhibitory actions of apelin on these neurons. Antagonistic regulation of apelin and vasopressin has a biological purpose, making it possible to maintain the water balance of the organism by preventing additional water loss via kidneys. This reveals a new physiological concept of dual and opposite functional potentiality for endocrine neurons coexpressing different neuropeptides in separate vesicles: depending on the degree of their electrical activation/inhibition, neurons release selectively the very coexpressed peptides that will ensure its accurate endocrine functions in perfect accordance with the hormonal demand.

Keywords: vasopressin; apelin; neuropeptides colocalization; diuresis; antidiuresis; water balance

Structure and processing of vasopressin and apelin

The neuropeptide arginine vasopressin (AVP) is a 9 amino acid peptide. It is synthesized and packaged

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DOI: 10.1016/S0079-6123(08)00443-3

in large, dense core vesicles within around 9000 magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, the axons of which project to the neurohypophysis. In these vesicles, AVP is associated tightly to the neurophysin II, a chaperone protein, an association playing a role during hormone biosynthesis. This prohormone is elaborated in Nissl bodies (Robert and Clauser, 2005). The hormone domain of the vasopressin prohormone is crucial for correct trafficking of the prohormone through the secretory pathway, and vasopressin–neurophysin association provides correct prohormone folding in the endoplasmic reticulum (De Bree et al., 2003). Vesicles are targeted to the different compartments of the AVP neurons, including the perikarya, dendrites and neurohypophysial nerve endings. At this later level, vesicles are densely stored, exocytosis occurring in response to neuronal action potentials. Vesicle content is then directly released into the systemic circulation where AVP exerts its various hormonal effects. The link between AVP and neurophysin II is broken up in the blood. In human, normally hydrated, the plasma concentration of AVP varied between 0.5 fmol/ml and 5 fmol/ml, the highest concentration occurring during the night (den Ouden and Meinders, 2005).

Apelin is a 36-amino acid peptide (apelin 36) generated from a larger precursor, the 77-amino acid, proapelin (Fig. 1). This precursor has been isolated from various species (Tatemoto et al., 1998; Habata et al., 1999a; Lee et al., 2000). The human proapelin gene is located on chromosome X at locus Xq25-q26.1 and contains three exons, with the coding region spanning exons 1 and 2. The 3′ untranslated region also spans two exons (2 and 3). This may account for the presence of transcripts of two different sizes (~3 kb and ~3.6 kb) in various tissues (Lee et al., 2000; O'Carroll et al., 2000). The alignment of proapelin amino acid sequences from cattle, humans, rats and mice has demonstrated strict conservation of the C-terminal 17 amino acids, known as apelin-17 or K17F (Fig. 1). In vivo, various molecular forms of apelin are present, differing only in length (either 36, 17 or 13 amino acids at the C-terminal part of the precursor). The occurrence of two internal dibasic motifs (Arg^{59}-Arg^{60}, Arg^{63}-Arg^{64}) within the cattle, human rat and mouse proapelin sequences suggests that K17F and pE13F may be processed by prohormone convertases. For apelin 36, the maturation mechanism remains to be defined. In rat lung, testis, uterus, and bovine colostrum, apelin 36 predominates, whereas both apelin 36 and pE13F have been detected in rat

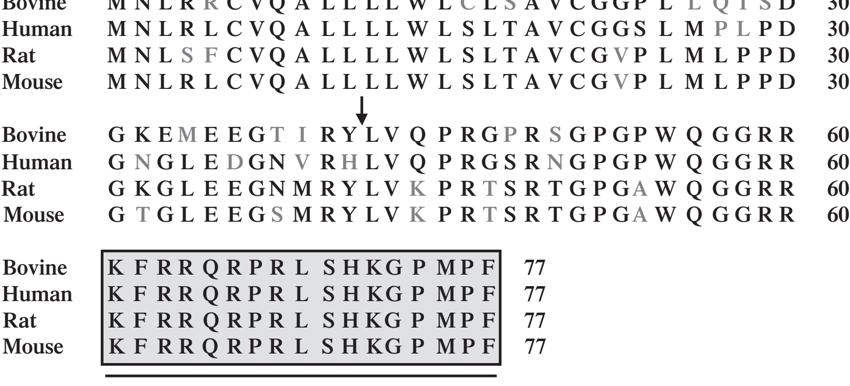

Fig. 1. Amino acid sequences of the apelin precursor, proapelin, in cattle, humans, rats and mice. The first amino acid of apelin 36 is indicated by an arrowhead and the apelin 17 (K17F) sequence is indicated by a grey box. Adapted with permission from Habata et al., 1999a and Tatemoto et al., 1998.

mammary gland (Hosoya et al., 2000; Kawamata et al., 2001). In rat brain and in rat and human plasma, the predominant forms of apelin are the pyroglutamyl form of apelin 13 (pE13F) and K17F (De Mota et al., 2004; Azizi et al., 2008). In healthy male subjects on a normal sodium diet, the plasma concentrations of apelin were 477 ± 167 fmol/ml (Azizi et al., 2008), whereas in rodents they were 3460 ± 250 fmol/ml (De Mota et al., 2004). As deduced from the transient hypotensive effect of K17F (2 min) when injected by the intravenous route in rodents (El Messari et al., 2004), a rapid metabolism of the apelin peptide may be postulated with a half life inferior to the minute.

Distribution of apelin and its receptor in the rat brain

The production of a polyclonal antibody with high affinity and selectivity for K17F (De Mota et al., 2004) has made it possible to visualize, for the first time, apelin neurons in the rat central nervous system. The precise central topographical distribution of apelin immunoreactivity shows that apelin-immunoreactive (IR) neuronal cell bodies are particularly abundant in the structures of the hypothalamus and medulla oblongata involved in neuroendocrine control, drinking behaviour and the regulation of arterial blood pressure, notably in the hypothalamic SON and the magnocellular part of the PVN, the arcuate nucleus, the lateral reticular nucleus and the nucleus ambiguus (Reaux et al., 2002). Conversely, apelin-IR nerve fibres are much more widely distributed in many brain regions than neuronal apelin cell bodies. The density of IR nerve fibres and apelinergic nerve endings is highest in the inner layer of the median eminence and in the posterior pituitary (Reaux et al., 2001; Brailoiu et al., 2002), suggesting that the apelin neurons of the SON and PVN, like the magnocellular AVP and oxytocin neurons, project into the posterior pituitary. Double immunofluorescence staining confirmed this finding, showing that apelin colocalized with AVP (De Mota et al., 2004; Reaux-Le Goazigo et al., 2004) and oxytocin (Brailoiu et al., 2002) in magnocellular hypothalamic neurons. Thus, apelin and AVP are synthetized from different genes in the same vasopressinergic magnocellular hypothalamic neurons. However, a marked segregation of apelin and AVP immunoreactivity within SON and PVN neurons was observed, suggesting that the two peptides might be stored in, and therefore differentially released from, two distinct vesicular pools within the same cells. This interpretation is further supported by the presence of apelin-positive/AVP-negative and AVP-positive/apelin-negative varicosities along the same hypothalamohypophyseal axons (De Mota et al., 2004; Reaux-Le Goazigo et al., 2004).

Like apelin, the apelin receptor (apelinR) is widely distributed throughout the rat central nervous system (De Mota et al., 2000; Lee et al., 2000; O'Carroll et al., 2000). In situ hybridization has shown that apelinR mRNA is present in the pyriform and entorhinal cortices, the septum, the hippocampus and structures containing monoaminergic neuronal cell bodies (pars compacta of the substantia nigra, dorsal raphe nucleus and locus coeruleus). The apelinR is particularly abundant in the apelin-rich hypothalamic nuclei, including the SON, PVN and the arcuate nucleus, and in the pineal gland and the anterior and intermediate lobes of the pituitary gland (De Mota et al., 2000). Furthermore, double labelling studies combining immunocytochemistry with in situ hybridization have demonstrated that, in the SON and PVN, apelinR (Reaux et al., 2001; O'Carroll et al., 2003), like the V1a- and V1b-type AVP receptor (AVPR, Hurbin et al., 1998), are synthesized by magnocellular AVP neurons suggesting an interaction between AVP and apelin.

Involvement of vasopressin and apelin in the maintenance of water balance

Action of central apelin on AVP neuron activity, systemic AVP release and diuresis

The neurosecretory neurons release AVP, an antidiuretic vasoconstrictor peptide, into the fenestrated capillaries of the posterior pituitary in

response to changes in plasma osmolality and volemia (Brownstein et al., 1980; Manning et al., 1977). The recent report of colocalization of AVP and apelin in magnocellular neurons of the hypothalamus and the presence of receptors for AVP and apelin on these neurons (Fig. 2) suggest a potential apelin response to these stimuli.

Regarding the involvement of apelin in the regulation of water balance, it is possible that, independently of the feedback control exerted by AVP on its own release, apelin may regulate AVP release. This hypothesis has been tested in lactating rats exhibiting a reinforced phasic pattern of AVP neurons during lactation, thereby facilitating systemic AVP release to maintain body-water content for optimal milk production. In this model, the i.c.v. injection of K17F inhibits the phasic firing activity of AVP neurons, thereby decreasing AVP release into the bloodstream, leading to water diuresis (Fig. 2; De Mota et al., 2004). These data suggest that apelin is probably released from the SON and PVN AVP cell bodies and inhibits AVP neuron activity and release by acting directly on the apelin autoreceptors

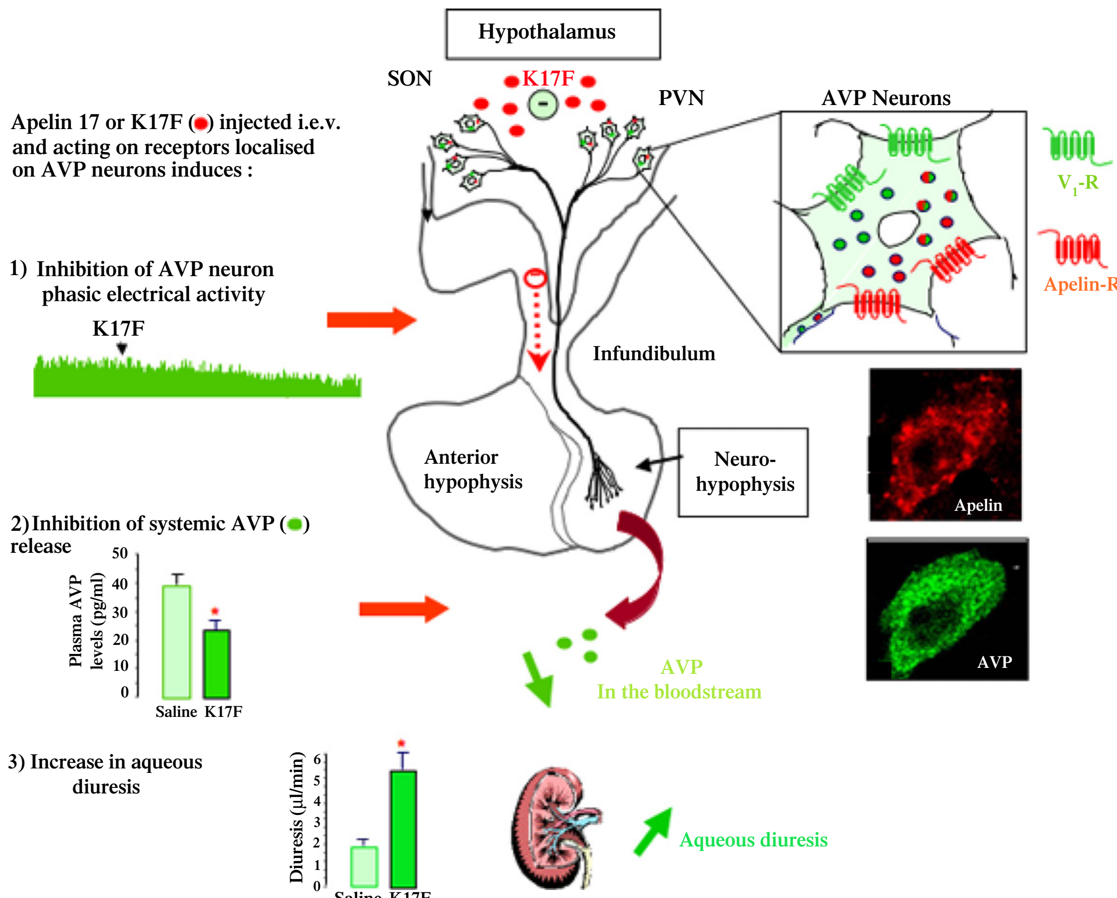

Fig. 2. Schematic diagram illustrating regulation of vasopressinergic magnocellular neurons by apelin. In rodents, apelin and its receptor are colocalized with AVP in the SON and PVN magnocellular neurons. In lactating animals, the central injection of apelin 17 induces gradual and sustained inhibition of the phasic electrical activity of AVP neurons, thereby decreasing systemic AVP secretion and increasing aqueous diuresis. Adapted with permission from De Mota et al., 2004, Reaux-Le Goazigo et al., 2004 and Reaux et al., 2001. (See Color Plate 43.2 in color plate section.)

expressed by AVP/apelin-containing neurons. This mechanism probably involves apelin acting as a natural inhibitor of the antidiuretic effect of AVP. The colocalization and opposite biological actions of these two peptides raises questions concerning how these two peptides are regulated to maintain body-fluid homeostasis. For this purpose, the effect of water deprivation on the neuronal content and release of both apelin and AVP were studied.

Opposite AVP and apelin expression and release following water deprivation

Following water deprivation, AVP is released in the blood circulation faster than it is synthesized, resulting in a depletion of AVP magnocellular neuronal stores (Fig. 3). Simultaneously, water deprivation decreases plasma apelin concentrations and induces a large increase in hypothalamic apelin neuronal content, especially that of the PVN and SON AVP magnocellular neurons (Fig. 3; De Mota et al., 2004; Reaux-Le Goazigo et al., 2004). It also resulted in the de novo appearance of apelin IR neurons in accessory magnocellular nuclei, which contain approximately one-third of oxytocin and AVP neurons projecting to the posterior pituitary (Reaux-Le Goazigo et al., 2004). Among these was the nucleus circularis, which, like the SON and PVN, receives inputs from the subfornical organ (SFO), a structure involved in the control of water balance. This suggests that, following water deprivation, apelin accumulates

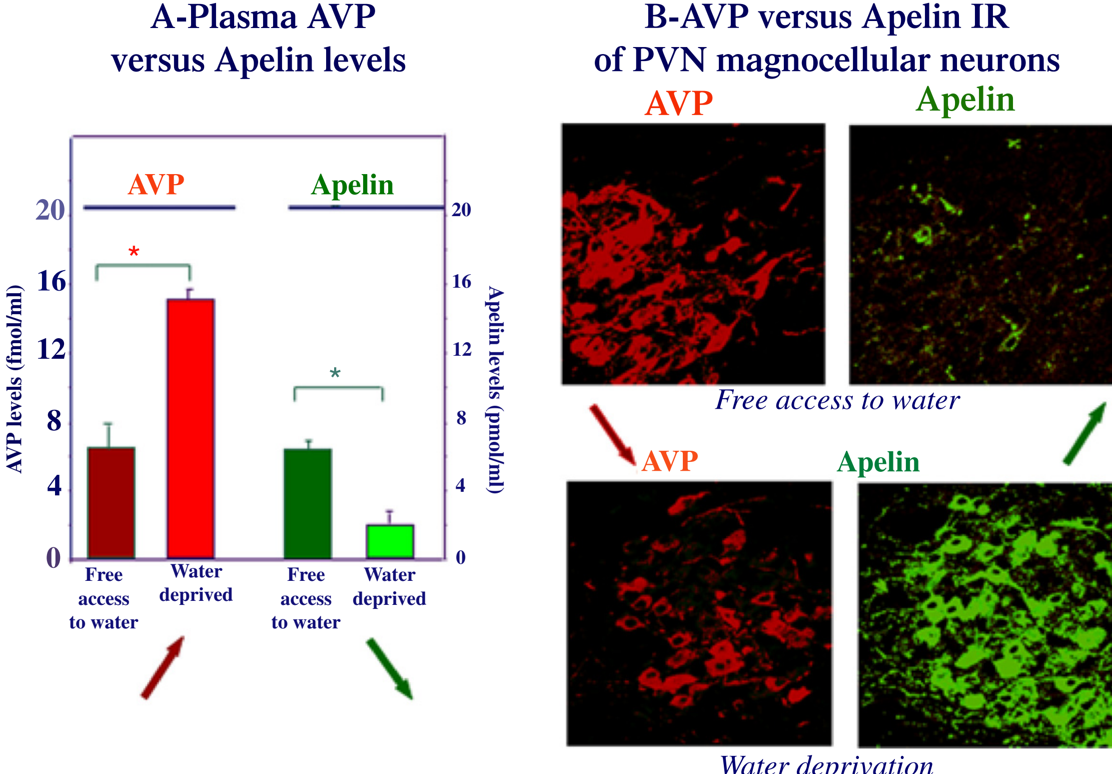

Fig. 3. Apelin and vasopressin are conversely regulated during water deprivation. In rats deprived of water, a large increase in apelin content of the PVN and SON magnocellular neurons is mirrored by a decrease in plasma apelin levels, suggesting that, under these conditions, apelin accumulates within AVP neurons rather than being released. Conversely, AVP content decreases within hypothalamic magnocellular neurons and increases in the bloodstream. Plasma apelin and AVP levels were measured by RIA. Confocal microscopic images of sections from the PVN immunoreacted against apelin (in green) and AVP (in red). Adapted with permission from De Mota et al., 2004 and Reaux-Le Goazigo et al., 2004. (See Color Plate 43.3 in color plate section.)

within AVP neurons rather than being released. The apelin response to dehydration is therefore the opposite of that of AVP, which is released faster than it is synthesized (Zingg et al., 1986; De Mota et al., 2004). This interpretation implies that apelin and AVP are released differentially by the magnocellular AVP neurons in which they are produced.

These opposite regulatory patterns of apelin and AVP suggest that these peptides act in concert to maintain body-fluid homeostasis. During dehydration, increases in the somatodendritic release of AVP optimize the phasic activity of AVP neurons (Gouzenes et al., 1998) facilitating the release of AVP into the bloodstream, whereas apelin accumulates in these neurons rather than being released into the bloodstream and, probably, into the nuclei. Thus, decreases in the local supply of apelin to the SON and PVN AVP cell bodies may facilitate the expression by AVP neurons of optimized phasic activity, by decreasing the inhibitory effects of apelin on these neurons. Antagonistic regulation of apelin and AVP has a biological purpose, making it possible to maintain the water balance of the organism by preventing additional water loss at the kidney level.

Vasopressin and apelin receptor subtypes involved in the control of AVP neuron activity

Concerning AVP autoreceptors, the V1a and V1b types (Hurbin et al., 1998), expressed in soma and dendrites, were shown to play an important role in regulating the activity of AVP neurons. The fact that these aureceptors are colocalized with AVP in the same vesicles (Hurbin et al., 2002) facilitates binding of the peptide to its autoreceptors when they are simultaneously released during exocytosis. The V1-type AVPR are coupled to a Gq protein which, when activated, will in turn activate a phospholipase C. Two molecular mechanisms can then be involved: either the mobilization of intracellular calcium via the activation of diacylglycerol (DAG); or the activation of a protein kinase C via the formation of inositol triphosphate (IP3, see references in review by Robert and Clauser, 2005). Both the resulting influx of calcium entry and the mobilization of intracellular calcium stores will have a determinant impact on the expression of the phasic pattern of AVP neurons.

The role and action mechanisms of apelin on its autoreceptors located on AVP neurons remain to be determined. However, multiple signalling pathways have been evidenced after activation of the apelinR stably expressed in eucaryotic cells. Rat and human apelinR stably expressed in Chinese hamster ovary (CHO) or human embryo kidney (HEK) cells are negatively coupled to adenylate cyclase activity and both are activated by several apelin fragments (Habata et al., 1999a, b; De Mota et al., 2000; Medhurst et al., 2003). The most potent inhibitors of forskolin-induced cyclic adenosine monophosphate (cAMP) production were found to be apelin 36, K17F, apelin 13 (Q13F) and pE13F, whereas shorter N-terminal deleted apelin fragments, R10F and G5F were inactive (Habata et al., 1999a; Medhurst et al., 2003; El Messari et al., 2004), Ala scan of pE13F (Medhurst et al., 2003) and N- or C-terminal deletions of K17F (El Messari et al., 2004) have shown that the arginine residues in positions 2 and 4 and the leucine residue in position 5 in pE13F play a critical role in binding affinity and in the inhibition of cAMP production. Apelin 36, K17F and pE13F also increase intracellular calcium mobilization in both NTera 2 human teratocarcinoma cells and RBL-2H3 cells derived from rat basophils stably expressing the human apelinR (Choe et al., 2000; Medhurst et al., 2003). Moreover, Masri et al. (2002) have also shown that pE13F activates extracellular signal-regulated kinases (ERKs) via a pertussis toxin-sensitive G protein and Ras-independent pathway. pE13F also activates p70 S6 kinase in human umbilical vein endothelial cells (HUVEC) and in CHO expressing the mouse apelinR, via both the ERK and phosphoinositide 3-kinase pathways (Masri et al., 2004). Apelin 36, K17F and pE13F induce internalization of the rat and human apelinR (Reaux et al., 2001; Zhou et al., 2003; El Messari et al., 2004), whereas deletion of the C-terminal phenylalanine of K17F abolishes internalization without affecting the adenylate cyclase coupling of the apelinR (El Messari et al., 2004). This interesting feature revealed that the apelinR G-protein coupling and internalization processes are dissociated, possibly reflecting the

existence of several conformational states of this apelinR, stabilized by the binding of different apelin fragments to the receptor (El Messari et al., 2004). This could account for different biological actions of these various apelin peptides produced in vivo.

Mechanism of vasopressin release and putative mechanism of apelin release

Frequency dependence of the release of coexisting neuropeptides

The existence of separate vesicle populations, containing only one or the other of the coexisting neuropeptide, implies that stimulus paradigms existing in vivo are used by the organism in vivo, and these paradigms permit selective release of the content of one but not the other vesicle population. The vesicle population storing apelin may attach to different releasing sites than those storing AVP. Also, AVP and apelin may be released at different frequencies of stimulation, yielding different intracellular calcium concentrations. Indeed, for any neuron in the nervous system, the amount of neurotransmitter (or neuropeptide) released by axon terminals at synaptic level depends on the arrival of action potentials and of their frequency. The calcium entry induced by action potentials induces the release of granules via a mechanism of exocytosis. The same applies for the hypothalamo-neurohypophysial neurons secreting neuropeptides directly into blood circulation via neurohemal junctions. It is well known that the amount of AVP released depends on the characteristics of phasic bursting activity (alternation of periods of stable electrical activity and silence), which mainly emerge when the demand of AVP release is increased (like during haemorrhage, dehydration). The activation is translated by an increased burst frequency or increased rates within bursts or both (see review by Hatton, 1990). Phasic bursting is more effective on AVP release per action potential basis, and during a burst, the release per pulse is greatest early in the burst when the rate is high.

Concerning apelin release, nothing is known at the present time. One would expect that its release by axon terminals in the neurohypophysis also depends on the arrival of action potentials like for all neurons. However, apelin release in the blood circulation is increased, contrary to AVP release, under physiological conditions known to strongly depress the electrical activity of AVP neurons like water loading (Azizi et al., 2008). In these conditions, AVP neurons decrease their firing rate and loose their phasic pattern, firing irregularly at low frequency (Hussy et al., 2000).

The first hypothesis could be that apelin release does not require high frequency of action potentials or a specific spike patterning like for AVP release. Previous data have reported a differential release of neuromediators coexpressed by the same neurons, according to the frequency of stimulation. This was the case for neurons expressing a classical neurotransmitter and a neuropeptide [like acetylcholine (Ach) and vasoactive intestinal peptide (VIP) in cerebral cortex neurons or norepinephrine (NE) and neuropeptide Y (NPY) in splenic nerve]: low-frequency stimulations release the neurotransmitter whereas peptide release requires, generally, higher frequencies of stimulation, or even a bursting pattern of stimulation (see review by Bartfai et al., 1988).

Such an observation also raises the possibility of a constitutive vs. a regulated secretion of apelin, as demonstrated for insulin (Ma et al., 1995). Constitutive secretion of insulin has been shown to be acutely sensitive to calcium: adding calcium inhibits secretion whereas removing calcium allows insulin secretion to return to its basal level. One can hypothesize that release of AVP that implies high frequency discharge and calcium entry, consequently lead to increasing intracellular calcium concentration, thus preventing apelin release. Also, it remains to demonstrate whether AVP and apelin releases depend or not on peptide aggregation as previously demonstrated for atrial natriuretic peptide (ANP, Newman and Severs, 1996). So, understanding the mechanisms allowing the preferential release of AVP or apelin would be of particular interest, especially as the physiological consequence of such differential releases, either regulated (owing to frequency coding) or constitutive,

would be a fine tuning and subtile regulation of water homeostasis through the selective release of one or the other neuropeptides required to ensure either diuresis (apelin) or the antidiuresis (AVP) according to the physiological conditions.

Retrocontrol of AVP neuron activity

The hypothalamo-neurohypophysial complex is not a simple output system releasing AVP into blood circulation, as AVP peptide is also released by soma and dendrites in the SON-PVN under many physiological conditions (see review by Ludwig, 1998). This dendritic exocytosis (Morris and Pow, 1988; Pow and Morris, 1989), induced by many transmitters and neuropeptides present in nerve fibres terminating in the SON-PVN (Ludwig et al., 1997), does not occur in conjunction with the release from axon terminals (see review by Ludwig, 1998). It first requires the relocation of peptide pools closer to sites of secretion, a mechanism selectively regulated through activation of intracellular calcium stores (Tobin et al., 2004). After priming, vesicles can subsequently be released by electrical and depolarization-dependent activation (Ludwig et al., 2002, 2005). This dendritic release has a key functional significance, as it regulates in turn the electrical activity of AVP neurons by favouring the expression of the phasic pattern known to be the most efficient for hormone release (Gouzenes et al., 1998). These data lead to the concept of modulation of neuronal activity by centrally released peptides: the optimization of a neuronal discharge in accordance with the physiological demand. Optimization of neuronal firing is attained through an autocrine mechanism since AVP neurons express autoreceptors of V1a and V1b types (Gouzenes et al., 1998). This "post-synaptic" control is complemented by a presynaptic action of AVP on afferent inputs (Kombian et al., 1997, 2002; Pittman et al., 2000), in particular the GABA ones via the V1a AVPR (Hermes et al., 2000). These modulatory actions would be particularly prominent in physiological situation increasing local AVP release.

Indeed, in response to osmotic stimulation, there is an increase in the somatodendritic release of AVP from SON and PVN cell bodies (Ludwig, 1998), enhancing, via the V1 autoreceptors, the phasic activity pattern of AVP neurons (Gouzenes et al., 1998) and thereby facilitating systemic AVP release.

Similarly, apelin seems to be involved in the autocrine somatodendritic feedback regulation of AVP neuron activity. In lactating rats exhibiting a reinforced phasic pattern of AVP neurons, the i.c.v. injection of K17F inhibits, via its autoreceptors located on AVP neurons, the phasic firing activity of these neurons, thereby decreasing AVP release into the bloodstream, leading to aqueous diuresis (Fig. 2; De Mota et al., 2004). Similarly, a marked decrease in systemic AVP release is observed following the i.c.v. injection of K17F or pE13F in mice deprived of water for 24 h (Reaux et al., 2001), a condition known to increase AVP neuron activity. Similarly, AVP could regulate apelin release: the dehydration-induced increase in apelin content in magnocellular AVP neurons is markedly diminished by the central injection of a selective V1 AVPR antagonist and is mimicked by the central infusion of AVP (Reaux-Le Goazigo et al., 2004). This suggests that the dehydration-induced accumulation of apelin in magnocellular neurons is caused by the somatodendritic release of AVP, which, by acting on the V1 AVPR, inhibits apelin release. Whether these retrocontrols are complementary or not, and are temporarily dissociated or not, remains to be determined.

A final feedback regulation by colocalized peptides is likely exerted in the neurohypophysis. Indeed, at axon terminal level, AVP and apelin are packaged in different vesicles, which suggests that AVP and apelin may also exert retrocontrol on their own release and/or the release of their coexisting neuropeptides.

Involvement of vasopressin and apelin in the maintenance of water balance at the kidney level

At the kidney level, the antidiuretic effect of AVP is mediated by V2 AVPR expressed in collecting

tubules (Terada and Marumo, 1993; Morello and Bichet, 2001), through the activation of a G-protein (Butlen et al., 1978; Jard et al., 1984). The $G\alpha_s$ protein activates a membrane adenylate cyclase, responsible for the intracellular production of cAMP, which itself activates a cAMP-dependant protein kinase A (PKA) (Orloff and Handler, 1967). Once activated in the cells of the collecting tubules, PKA ensures water retention through two mechanisms. On the one hand, PKA phosphoryles the water channels of aquaporine 2 (AQP2), localized in small intracellular vesicles. This phosphorylation allows the translocation of AQP2 to the apical membrane, and increases the number and function of these channels. On the other hand, PKA phosporyles the transcription factor, cAMP response element binding protein (CREB), responsible for AQP2 gene transcription (see review by Robert and Clauser, 2005).

Consistent with a renal action of apelin, apelinR, proapelin mRNA and apelin peptide have been detected in the rat and human kidney (O'Carroll et al., 2000; Medhurst et al., 2003; Kleinz and Davenport, 2004), and apelinR mRNA has been shown to be particularly abundant in vasa recta in the inner stripe of the outer medulla, a region known to play a key role in water and sodium balance. These data suggest that apelin may play a crucial role in the maintenance of body-fluid homeostasis by counteracting AVP actions not only at the central but also at the renal level.

Conclusion

In conclusion, the experimental data obtained to date show that apelin is colocalized with AVP, V1 AVPR and apelinR in hypothalamic magnocellular neurons. They demonstrate that apelin, by inhibiting the phasic electrical activity of these neurons and the systemic secretion of AVP induces water diuresis. Overall, these data show that this new circulating vasoactive neuropeptide may play, together with AVP, a key role in the maintenance of water balance. Moreover, water deprivation, which increases systemic AVP release and causes depletion of hypothalamic AVP stores, decreased plasma apelin concentrations and induced hypothalamic accumulation of the peptide, indicating that AVP and apelin are conversely regulated to facilitate systemic AVP release and avoid additional water loss at the kidney level. Altogether, this suggests a new physiological concept of dual potentiality for endocrine neurons that, according to the degree of their activation/inhibition will dynamically ensure opposite physiological functions in perfect accordance with the hormonal demand, owing to optimize the selective release of one of their coexpressed peptides. No bioavailable nonpeptide agonist or antagonist of the apelinR has yet been discovered, but obtaining such compounds would make it possible to highlight the intracellular mechanisms involved in apelin receptor activation in AVP neurons as well as to further explore the role played by this peptide further upstream in body-fluid homeostasis.

Abbreviations

Ach	acetylcholine
ANP	atrial natriuretic peptide
ApelinR	apelin receptor
AQP2	aquaporine 2
AVP	arginine vasopressin
AVPR	AVP receptor
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CREB	cAMP response element binding protein
DAG	diacylglycerol
ERK	extracellular signal-regulated kinase
HEK	human embryo kidney
HUVEC	human umbilical vein endothelial cells
IP3	inositol triphosphate
IR	immunoreactive
NE	norepinephrine

NPY	neuropeptide Y
PKA	protein kinase A
PVN	paraventricular nucleus
SON	supraoptic nucleus
VIP	vasoactive intestinal peptide

Acknowledgements

This work was funded by the Institut National de la Santé et de la Recherche Medicale (INSERM), the Société Française d'Hypertension Artérielle, the France–Hungary co-operation programme BALATON, the Fonds de la Recherche en Santé du Québec and the National Research Agency programme (ANR- 05- PCOD-001-01). I would particularly like to thank all those in the laboratory who contributed to these studies: N. De Mota, S. El Messari, C. Fassot, A. Hus-Citharel, X. Iturrioz, Z. Lenkei and A. Reaux-Le Goazigo. Much of the work presented here was carried out in collaboration with INSERM U413 (Prof. H. Vaudry), with the neuromorphology laboratory of Prof. M. Palkovits (Semmelweis University, Budapest, Hungary) and the functional neuroimaging laboratory of Prof. A. Beaudet (Montreal Neurological Institute, McGill University, Montreal, Canada).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 44

Recent discoveries in vasopressin-regulated aquaporin-2 trafficking

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Abstract: In the kidney, the actions of the antidiuretic hormone arginine vasopressin (AVP) renders the collecting duct highly permeable to water. This large increase in water permeability is largely due to the translocation of the water channel aquaporin-2 (AQP-2) from intracellular storage vesicles to the apical plasma membrane of collecting duct principal cells. The focus of this chapter is on the recent advances in interpreting the complex mechanism that causes regulated exocytosis of AQP-2 to the apical plasma membrane, its regulated endocytosis and the recycling of AQP-2. Determining how AQP-2 trafficking occurs at the molecular level is fundamental to understanding the physiology of water balance regulation and the pathophysiology of water balance disorders.

Keywords: aquaporin/s; urinary concentrating mechanism; microtubule; actin; PKA; phosphorylation

Aquaporins and the urinary concentrating mechanism

Aquaporins (AQPs) are a family of water channels that mediate movement of water (and other small solutes) across the cells' lipid bilayer along osmotic gradients. AQPs are expressed in almost all living organisms in numerous different tissues (Nielsen et al., 2002). In the mammalian kidney, at least eight AQPs have been localized to various segments of the renal tubule and play essential roles in the maintenance of body-water homoeostasis. The majority of fluid filtered by the glomerulus is reabsorbed by an active near-isosmolar transport mechanism in the proximal tubule by aquaporin-1 (AQP-1), which is localized to both the apical and basolateral plasma membranes of epithelial cells in the proximal tubule (Nielsen et al., 1993). In addition, AQP-1 is also expressed in the long, thin, descending limbs of the loop of Henle (lDL) and the epithelium of the descending vasa recta (DVR) (Nielsen et al., 1995c, 2002), nephron segments thought to be involved in countercurrent multiplication and exchange. The constitutively high water permeability of nephron segments expressing AQP-1 is consistent with the lack of regulation of AQP-1 by vasopressin (Terris et al., 1996).

In contrast to the constitutively high water permeability of the proximal tubule, the collecting duct (CD) has relatively low water permeability, and only upon stimulation by arginine vasopressin (AVP) or other hormones such as oxytocin (Chou et al., 1995) does the CD epithelium become highly permeable to water (Nielsen et al., 1995a). Indeed, the water permeability of the CD can increase by more than 10-fold after AVP stimulation, allowing for the osmotically driven movement of water from the lumen to the interstitium and allowing the

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DOI: 10.1016/S0079-6123(08)00444-5

kidney to produce highly hypertonic urine (less that 0.51 urine per day in humans) during periods of water deprivation. The majority of water transport across the CD epithelium occurs by a transcellular route with serial passage across the apical plasma membrane, mediated by AQP-2 and the basolateral plasma membranes, mediated by a combination of aquaporin-3 (AQP-3) and aquaporin-4 (AQP-4) (Nielsen et al., 2002).

The dramatic increase in CD water permeability upon AVP stimulation occurs in minutes and is largely due to the translocation of AQP-2 from intracellular storage vesicles to the apical plasma membrane of CD principal cells (Nielsen et al., 1995a). This trafficking event leads to accumulation of AQP-2 in the plasma membrane.

The essential role that AQP-2 plays in the urinary concentrating mechanism is underlined in numerous clinical disorders that result in defects in body-water balance (Schrier and Cadnapaphornchai, 2003). In several of these conditions, dysregulated AQP-2 trafficking is observed. For example, several mutations in the AQP-2 gene can cause the rare disorder of hereditary nephrogenic diabetes insipidus (NDI, autosomal recessive or dominant) (reviewed in Fujiwara and Bichet, 2005). These mutations result in misfolding of AQP-2 and retention in, for example, the endoplasmic reticulum (ER), the Golgi complex or late endosomes. In addition, numerous pathophysiological disorders result in defective AQP-2 trafficking (Nielsen et al., 2002).

Targeting mechanisms

To completely understand how regulated AQP-2 trafficking occurs, it is necessary to understand that several different pathways combine for the insertion of AQP-2 into the apical plasma membrane. Signal transduction pathway analysis is required to elucidate how downstream events, such as binding of AVP to the type II vasopressin receptor in the CD (Fenton et al., 2007), trigger a cascade of intracellular responses and result in 'mobilization' of AQP-2 containing vesicles. For example, it has been clearly shown that phosphorylation of AQP-2 occurs intracellularly and is essential for the transport of AQP-2 from intracellular stores to the apical membrane (described later). In addition, studies of how AQP-2 vesicles are physically trafficked to the apical membrane requires knowledge of the complex field of vesicle transport. In general, after synthesis in the ER, the majority of proteins are transported through the Golgi apparatus and the trans-Golgi network (TGN) before being packaged into cargo vesicles and subsequently distributed to different cellular compartments. In the case of AQP-2, this is predominantly the apical plasma membrane; thus, it is important to discover if AQP-2 contains sequence motifs that interact with components of the cellular sorting machinery and determine its final destination.

For the purposes of this review, the transport of AQP-2 from the ER to the membrane is considered as two distinct steps: (1) transport from the ER to sub-apical storage vesicles and (2) fusion of these storage vesicles with the apical plasma membrane (exocytosis). Despite the portrayal of a simplistic mechanism in this chapter, other routes such as direct transport of AQP-2 containing vesicles to the apical plasma membrane may also occur. Furthermore, it is important to consider that the accumulation of AQP-2 on the apical plasma membrane is a balance between regulated exocytosis and endocytosis, inhibition of which also increases the osmotic water permeability of the CD (Sun et al., 2002; Lu et al., 2004). Despite more than 10 years of research into the AQP-2 trafficking 'phenomena', the complete picture of this complicated process is still being slowly resolved (see Fig. 1 for overview).

Effect of arginine vasopressin

In response to small increases in plasma osmolality or a reduction in the effective circulating blood volume, AVP is released by the posterior pituitary gland and promotes water reabsorption in the kidney's collecting duct, promotes enhance sodium reabsorption in the thick ascending limb (TAL) and vasoconstriction via four subtypes of receptors (Holmes et al., 2003). The antidiuretic effects of AVP in the CD result from a cascade of events, initialized by the binding of AVP to the type 2

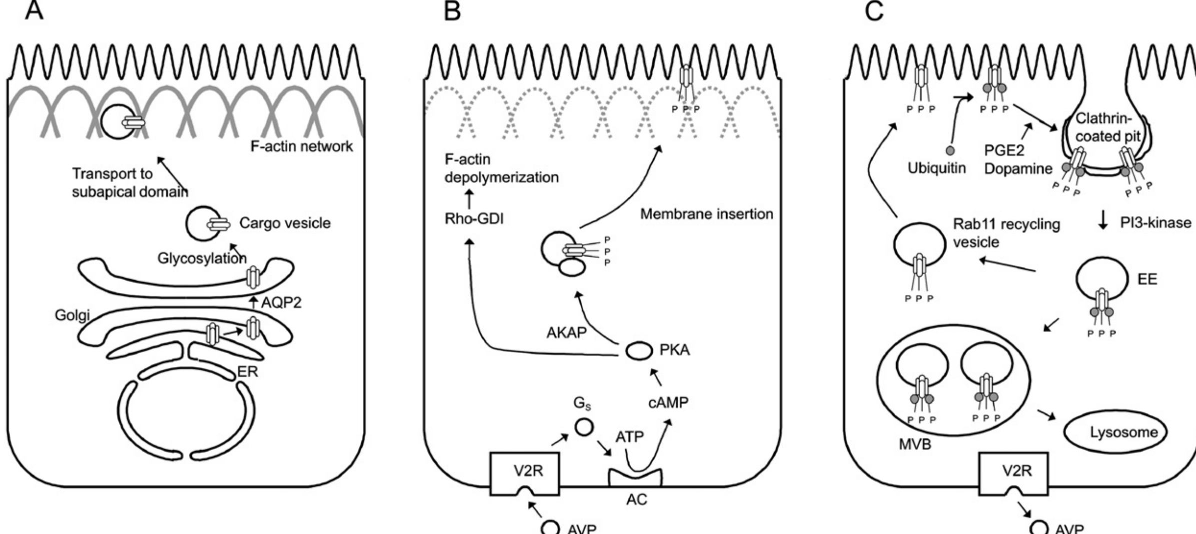

Fig. 1. Schematic illustration of AQP-2 trafficking in the collecting duct's principal cell. (A) AQP-2 synthesis in the endoplasmic reticulum (ER) and transport through the Golgi apparatus to a final localization in the subapical domain, where AQP-2 vesicles are held in place by the F-actin network. (B) Vasopressin induced elevation of adenosine 3′,5′-cyclic monophosphate (cAMP) and activation of protein kinase A (PKA). PKA is recruited to AQP-2 vesicles via PKA-anchoring proteins (AKAPs). This leads to phosphorylation of AQP-2 and insertion into the apical membrane. PKA activation also leads to stabilization of the inactive form of Rho (Rho-GDI) resulting in apical actin depolymerization favouring vesicle fusion with the membrane. (C) Endocytic retrieval of AQP-2 initiated by removal of AVP or favoured by prostaglandin E2 (PGE2) or dopamine. Ubiquitination takes place in the membrane and may play a role in the subsequent endocytotic events. Endocytosis of AQP-2 occurs in clathrin-coated pits via a phosphatidylinositol 3-kinase (PI3-kinase)-dependent mechanism. AQP-2 is transported to either early endosomes (EE) or recycling vesicles or to multivesicular bodies (MVB) targeted for proteasomal degradation. AVP, antidiuretic hormone; V2R, vasopressin receptor; Gs, GTP-binding protein; AC, adenylate cyclase; ATP, adenosine triphosphate; P indicates phosphorylation.

vasopressin receptor (V2). This results in, amongst other things, activation of adenylate cyclase through the heterotrimeric guanine triphosphate (GTP)-binding protein Gs, an increase in intracellular cAMP, activation of protein kinase A (PKA) and phosphorylation of AQP-2 (described later).

In addition to increasing intracellular cAMP levels, binding of AVP to the V2 results in increased intracellular Ca^{2+} levels (Star et al., 1988; Ecelbarger et al., 1996), a result of the calmodulin-dependent release of Ca^{+} from ryanodine-sensitive intracellular stores (Chou et al., 2000). Inhibiting the AVP-induced calcium response prevents AQP-2 trafficking but has no effect on intracellular cAMP levels in isolated perfused tubules (Chou et al., 2000), indicating that calcium is necessary for AQP-2 exocytosis. It has recently been shown that activation of exchange protein directly activated by cAMP (EPAC) triggers intracellular Ca^{2+} mobilization and apical exocytotic insertion of AQP-2 in the CD (Yip, 2006).

In addition to AVP-dependent mechanisms, a number of other regulatory mechanisms may be involved in regulating AQP-2 trafficking. As mentioned earlier, the hormone oxytocin increases collecting duct water permeability and has an antidiuretic effect (Chou et al., 1995). In addition, cGMP pathways may be involved in AQP translocation. The carboxyl-terminal tail of AQP-2 is a target for phosphorylation by protein kinase G (PKG) and both nitric oxide and atrial naturetic peptide, which can increase cGMP levels, induce AQP-2 trafficking to the plasma membrane, without a resultant rise in intracellular cAMP (Bouley et al., 2000). However, it is not completely clear whether AQP-2 can be directly phosphorylated by PKG, or whether the effects observed are mediated by a subsequent activation of PKA.

Role of phosphorylation

There are several putative phosphorylation sites for different kinases in the AQP-2 amino acid sequence, including PKA and PKG, PKC, Golgi casein kinase and casein kinase II sites. Thus far, the majority of research has focused on the role of PKA-induced phosphorylation on AQP-2 trafficking. The rise in intracellular cAMP following activation of G-coupled proteins by AVP results in recruitment of PKA to AQP-2-containing vesicles by PKA-anchoring proteins (AKAPs) (Klussmann et al., 1999). Intracellularly, the co-localization of vesicular AQP-2 with AKAP 18 delta makes this the most likely isoform to mediate this event (Henn et al., 2004). Furthermore, it has recently been shown that, inhibition of the cAMP-specific phosphodiesterase-4D (PDE4D) with rolipram increases AKAP-tethered PKA activity in AQP-2-bearing vesicles and enhances AQP-2 trafficking (Stefan et al., 2007), indicating that a novel, compartmentalized cAMP-dependent signal transduction pathway consisting of anchored PDE4D, AKAP18delta and PKA plays an essential role in AQP-2 translocation.

Following PKA recruitment, phosphorylation of AQP-2 on serine 256 (S256) has been shown to be critical for vasopressin-induced cell-surface accumulation of AQP-2 (Fushimi et al., 1997; Katsura et al., 1997). However, using phosphospecific antibodies and immunoelectron microscopy, it was clearly shown that the S256 phosphorylated form of AQP-2 is localized both intracellularly and in the plasma membrane (Christensen et al., 2000), indicating that it is constitutively phosphorylated, even in low circulating vasopressin states. Further studies indicated that after AVP stimulation pS256-AQP-2 translocates to the apical plasma membrane where AQP-2 exists as a tetramer, with at least three monomers in an AQP-2 tetramer being phosphorylated (Kamsteeg et al., 2000). Indeed, although the majority of studies have been performed in cell-culture models or oocytes, a mouse strain has recently been identified with an amino-acid substitution at S256, preventing phosphorylation of this residue, and manifesting in congenital progressive hydronephrosis due to major polyuria. This highlights the essential role of S256 phosphorylation in AQP-2 trafficking and the urinary concentrating mechanism (McDill et al., 2006). In addition to S256, phosphoproteomics studies have recently discovered that AQP-2 is further phosphorylated on residues S261, S264 and S269 (Hoffert et al., 2006) in response to AVP stimulation, and immunolocalization studies suggest that these phosphorylated forms are localized to different intracellular compartments (Hoffert et al., 2007; Fenton et al., 2008). The role that these additional phosphorylation sites play in both AQP-2 trafficking and CD water permeability will likely be a focus of future research.

Despite clear evidence that phosphorylation of AQP-2 is required for cell surface expression, it still remains unclear as to how phosphorylation of AQP-2 at serine 256 (or S261, S264 and S269) *actually* induces apical trafficking. One possibility is that phosphorylation itself could directly influence an interaction between AQP-2 containing vesicles and the cell cytoskeleton, microtubules or accessory cross-linking proteins (described later), examination of which should be possible in the current ‘proteomics era’. Indeed, it has recently been shown that S256 is important for a direct interaction of AQP-2 with 70 kDa heat shock proteins and, ultimately, the AQP-2 shuttle (Lu et al., 2007). Another possible mechanism is that phosphorylation could prevent endocytosis of AQP-2, leading to accumulation at the cell surface.

Although phosphorylation of AQP-2 is usually required for cell surface expression, the internalization of AQP-2 is not dependent on its phosphorylation state. In support of this, prostaglandin E2 can promote removal of AQP-2 from the cell surface after AVP stimulation without altering the phosphorylation state of AQP-2 (Tamma et al., 2003b) and, in transfected cells, both prostaglandin E2 and dopamine can induce internalization of AQP-2 independently of AQP-2 dephosphorylation (Nejsum, 2005).

Although PKA is likely to play a major role in AQP-2 translocation, there is evidence that both other kinases and PKA-independent mechanisms may also be involved. Increased AQP-2 at the cell surface has been demonstrated in the presence of the phosphatase inhibitor okadaic acid, even in the presence of the specific PKA inhibitor H89. Ser256

is also a substrate for Golgi casein kinase 2, and phosphorylation by this kinase is required for the Golgi transition of AQP-2 (Procino et al., 2003). Furthermore, S256A-AQP-2 mutants (that lack S256 phosphorylation) are able to accumulate at the cell surface after treatment with cholesterol-depleting agents (described later) (Lu et al., 2004), and it has been shown that activation of the PKC pathway mediates AQP-2 endocytosis independently of phosphorylation state (Van Balkom et al., 2002).

Role of the cytoskeleton and associated proteins

Actin

The regulatory role of the actin cytoskeleton in AQP-2 trafficking has been extensively investigated, and a direct binding of AQP-2 to actin has been demonstrated (Noda et al., 2004). Furthermore, both β- and γ-actin have been identified in kidney inner medulla in association with AQP-2-containing vesicles (Barile et al., 2005). Early studies conducted in rat inner medulla and toad urinary bladder demonstrated that AVP induces a reduction in F-actin in apical regions of cells (Ding et al., 1991; Hays et al., 1993) and that F-actin depolymerizing agents, such as cytochalasins, inhibit responses to AVP (Wade and Kachadorian, 1988). Importantly, the pool of actin depolymerized by AVP appears to overlap with the pool depolymerized by cytochalasin. Although these results appear conflicting (AVP increases water permeability by depolymerization of actin whereas cytochalasin decreases osmotic water permeability), they do suggest a dual role for actin: one to serve as a vesicle holding network that allows membrane fusion upon AVP stimulation and the other to transport AQP-2 vesicles to the apical membrane (Franki et al., 1992). Furthermore, depolymerization of the cortical actin network could depend, in part, on calcium-dependent actin-serving enzymes such as gelsolin, which has been found to be associated with AQP-2 (Noda et al., 2005).

More recently, it has been shown that actin depolymerization is a prerequisite for cAMP-dependent translocation of AQP-2 to the plasma membrane (Valenti et al., 2000), and that regulators of the actin cytoskeleton, such as the Rho family of small GTP-binding proteins (Rho, Rac and Cdc42), influence AQP-2 translocation via regulation of F-actin-containing cytoskeletal structures (Klussmann et al., 2001; Tamma et al., 2001). Indeed, selective inhibition of Rho is sufficient for the depolymerization of actin, AQP-2 translocation and increased water permeability, even in the absence of AVP stimulation. It has been shown that AVP is able to modulate the negative effect of Rho on AQP-2 trafficking by stabilizing its inactive form, Rho-GDI, by a PKA-mediated event (Tamma et al., 2003a). Furthermore, ezrin/radixin/moesin (ERM) proteins, activated through a Rho signalling mechanism may also be involved in organization of the cortical actin cytoskeleton (Tamma et al., 2005) and thus AQP-2 translocation. In addition to directly influencing trafficking, actin may also play a role in membrane retrieval of AQP-2 and its subsequent transfer to subapical compartments, as actin filament-disrupting agents result in accumulation of AQP-2 in both early and recycling endosomes (Tajika et al., 2005).

Microtubules

In rat kidney, cold-induced disruption of microtubules or treatment with colchicines results in scattering of AQP-2 vesicles and inhibited AQP-2 trafficking to the plasma membrane (Sabolic et al., 1995; Breton and Brown, 1998). Upon AVP stimulation, it has been shown that the microtubule network that is usually densely formed around the cell nucleus is reversibly reorganized with increased formation of microtubules in the periphery of the cells (Vossenkamper et al., 2007). Whether microtubules affect endocytic retrieval or the exocytic pathway of AQP-2 remains unclear. However, elegant studies by Vossenkamper et al. (2007) clearly show that depolymerization of microtubules prevents the perinuclear positioning of AQP-2 in resting cells and, after internalization of AQP-2 following AVP washout, forskolin stimulation can still cause a redistribution of AQP-2 to the plasma membrane, even in the presence of microtubule-disrupting agents. Taken together, these results suggest that the microtubule-dependent translocation of AQP-2

is predominantly responsible for trafficking and localization of AQP-2 inside the cell after internalization but not for its exocytic translocation.

Motorproteins

Vesicular transport is partly dependent on motor proteins that move along microtubules or microfilaments, and a recent study suggests that several proteins (e.g. SPA-1, myosin regulatory light chain smooth muscle isoforms 2-A and 2-B, annexin A2 and annexin A6) interact directly/indirectly with AQP-2 and form a multiprotein motor complex that is essential for AQP-2 trafficking (Noda et al., 2005). Early studies showed that the microtubule-associated motor protein dynein is present on AQP-2-bearing vesicles (Marples et al., 1998), although recently a large-scale proteomics study did not find dynein in association with AQP-2 bearing vesicles (Barile et al., 2005). However, inhibition of dynein can impair AQP-2 transport to the perinuclear space in a way similar to microtubule disruption, suggesting that dynein does play a role in AQP-2 trafficking.

Other forms of motor proteins, myosins, may also be important in AVP-regulated water permeability. Myosin heavy chains (corresponding to myosin IIA and II B), myosin light chain (MLC), myosin light chain kinase (MLCK) and phosphorylated forms of MLC have all been localized to the kidney inner medulla. Furthermore, Chou et al. (2004) have shown that AVP increases MLC phosphorylation (by MLCK) and that inhibition of MLCK inhibits osmotic water permeability in isolated perfused tubules. Coupling of motor proteins to vesicles requires multiple Rab proteins, and recently myosin Vb has been suggested to play a role in the AQP-2 shuttle by a Rab11-FIP2-dependent recycling through a perinuclear Rab11 compartment (Nedvetsky et al., 2007).

Docking machinery

Docking and fusion of AQP-2-containing vesicles with the plasma membrane may involve vesicle soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (v-SNAREs) and target membrane (t) SNARES. The SNARE protein VAMP-2 (vesicle associated membrane polypeptide-2, also known as synaptobrevin-2) has been found associated with AQP-2 vesicles (Nielsen et al., 1995b; Jo et al., 1995), and disruption of VAMP-2 with tetanus toxin diminishes cAMP-dependent AQP-2 trafficking (Gouraud et al., 2002). Additionally, SNARES that co-localize with AQP-2 include VAMP-3 (cellubrevin), SNAP23 (synaptosomal associated protein 23) and the ATPase Hrs-2 (Franki et al., 1995; Inoue et al., 1998; Shukla et al., 2001). The t-SNARE syntaxin-4 is present in the apical plasma membrane of collecting duct principal cells (Mandon et al., 1996), and SNAP-23 has been found to be associated with syntaxin-4 and VAMP-2 (Ravichandran et al., 1996). Furthermore, members of the Rab GTPase family Rab3 and Rab5a (Liebenhoff and Rosenthal, 1995) have been localized to AQP-2-containing vesicles and thus may also play a role in vesicle docking and fusion. Taken together, although numerous vesicle docking and fusion proteins associate with AQP-2, their importance remains undetermined.

Role of endocytosis

As mentioned earlier, the accumulation of AQP-2 on the apical plasma membrane of collecting duct principal cells is a balance between regulated exocytosis and endocytosis. During the endocytic process, AQP-2 accumulates in clathrin-coated pits before being internalized via a clathrin-mediated process (Lu et al., 2004; Bouley et al., 2006; Russo et al., 2006). Internalization of AQP-2 is not dependent on its phosphorylation state (see ‘Role of Phosphorylation’). Once internalized, a phosphatidylinositol-3-kinase-dependent mechanism retrieves AQP-2 to EEA1-positive early endosomes, before AQP-2 is transferred to Rab11-positive storage vesicles (Tajika et al., 2004). Upon re-stimulation with AVP, AQP-2 may be recycled to the apical plasma membrane, a process that is thought to involve the protein Rab11 (Barile et al., 2005; Tajika et al., 2005). Although the signal for endocytosis remains unclear, recent evidence suggests that ubiquitination is important (Kamsteeg et al., 2006). AQP-2 is ubiquitinated in vivo, and this likely

occurs on the plasma membrane. Once ubiquitinated, AQP-2 can be readily internalized where it is transported to multivesicular bodies (MVBs) and targeted for proteasomal degradation. A proportion of AQP-2 that is internalized to MVBs can be re-excreted into the urine as exosomes (Pisitkun et al., 2004).

Future perspectives

Numerous studies over the past several years have resulted in considerable progress in our understanding of AQP-2 translocation. Continuing investigations will hopefully provide the detailed molecular mechanisms behind this complex 'phenomena' and thus provide invaluable insight into the fundamental physiology and pathophysiology of water-balance homoeostasis.

Acknowledgements

The authors thank Dr. Jeppe Praetorius for critical reading of the manuscript. Robert A. Fenton is supported by a Marie Curie Intra-European Fellowship, the Carlsberg Foundation (Carlsberg-fondet) and the Danish National Research Foundation (Danmarks Grundforskningsfond). The Water and Salt Research Center at the University of Aarhus is established and supported by the Danish National Research Foundation (Danmarks Grundforskningsfond).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 45

Pathophysiological role of aquaporin-2 in impaired water excretion

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Abstract: In a state of chronic arginine vasopressin (AVP)-induced antidiuresis, the antidiuretic efficacy has been attenuated: a phenomenon known as "AVP escape". We compared the experimental SIADH rats with 1-deamino-8-D-AVP (dDAVP)-excess rats. The SIADH rats, but not the dDAVP-excess rats, showed a marked attenuation of urinary concentrating ability. This is closely associated with diminished up-regulation of aquaporin-2 (AQP-2) mRNA and protein expression. The following in vitro study clarified tonicity-response elements in the 5′-flanking region of AQP-2 gene. There are at least more than two hypertonicity-response elements, and a hypotonicity-response element resided at tonicity-response enhancer (TonE) (−570 to −560 bp) in the AQP-2 gene. Hypotonicity directly reduced the cAMP-induced AQP-2 promoter activity by mediating JNK kinase. Reduction in transcriptional regulation of AQP-2 under hypotonic state may support the in vivo finding of AVP escape phenomenon in chronic AVP-induced antidiuresis.

Keywords: aquaporin-2; arginine vasopressin; tonicity; gene expression; promoter activity

Pathological states of water retention

Clinical and laboratory findings have demonstrated that impaired water excretion occurs in pathological states of euvolemic and hypervolemic hyponatremia (Schrier, 1988a, b; Ishikawa and Schrier, 2003). Euvolemic hyponatremia is found in syndrome of inappropriate secretion of antidiuretic hormone (SIADH), glucocorticoid deficiency and mineralocorticoid-responsive hyponatremia of the elderly (MRHE) (Bartter and Schwartz, 1967; Ishikawa et al., 2001; Yatagai et al., 2003). Also, edematous diseases including congestive heart failure, liver cirrhosis with ascites and nephrotic syndrome cause hypervolemic hyponatremia (Schrier, 1988a, b). In these clinical settings non-suppressible release of arginine vasopressin (AVP) is found despite hypoosmolality, which should reduce AVP release to undetectable levels. The exaggerated release of AVP is inappropriate in SIADH and edematous diseases. Particularly, in the edematous diseases it is evidence for an increase in body water and sodium, but a decrease in effective circulatory blood volume causes non-osmotic release of AVP (Schrier and Abraham, 1999). In contrast, enhanced release of AVP is appropriate in MRHE (Ishikawa et al., 2001).

In response to AVP urine is concentrated by water reabsorption across the renal collecting ducts (Knepper and Rector, 1995; Ishikawa and Schrier, 2003). Sasaki et al. (Fushimi et al., 1993; Sasaki et al., 1994) recently cloned a complementary DNA of apical collecting duct water channel

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DOI: 10.1016/S0079-6123(08)00445-7

aquaporin-2 (AQP-2) from rat and human kidney. AQP-2 is an AVP-regulated water channel. AVP translocates AQP-2 from cytoplasmic vesicles to apical plasma membranes, which is called short-term regulation, and increases the expression of AQP-2 mRNA in collecting duct cells. A cAMP-responsive element is located at −310 through −304 bp of the 5′-flanking region of the AQP-2 gene. Because AVP increases cAMP concentration in target cells, it is highly likely that AVP regulates the transcription of the AQP-2 gene in the collecting duct (long-term regulation).

Urinary AQP-2 was detected in both soluble and membrane-bound forms by western blot analysis (Kanno et al., 1995). Immunoblots of urine samples showed AQP-2 with molecular sizes of 29 and 40–50 kDa. The band at 40–50 kDa represents a glycosylated form of the 29 kDa protein. Immunoelectron microscopy demonstrated immunogold labelling with a specific antibody against AQP-2 along with the membrane structure-forming vesicle-like shapes in the urinary sediments of high-speed centrifugation (12,000*g*). Radioimmunoassay for AQP-2 allows a quantitative analysis of the urinary excretion of AQP-2. Rai et al. (1997) estimated that the fraction of AQP-2 excreted into urine in the rat was approximately 3% of the AQP-2 presented in renal collecting duct cells. A positive correlation was found between urinary excretion of AQP-2 and plasma AVP levels (Saito et al., 1997) and urinary excretion of AQP-2 and urinary osmolality (Rai et al., 1997). There is a marked difference in urinary excretion of AQP-2 in the disorders of water metabolism. The urinary excretion of AQP-2 was 80% less in the patients with central diabetes insipidus compared to the control subjects. By contrast, the urinary excretion of AQP-2 was markedly increased in the patients with impaired water excretion. Augmentation in urinary excretion of AQP-2 is found in SIADH, hypopituitarism, MRHE, liver cirrhosis and heart failure (Ishikawa et al., 2001; Funayama et al., 2004).

Figure 1 shows urinary excretion of AQP-2 in the 65 patients with congestive heart failure (Funayama et al., 2004). Urinary excretion of

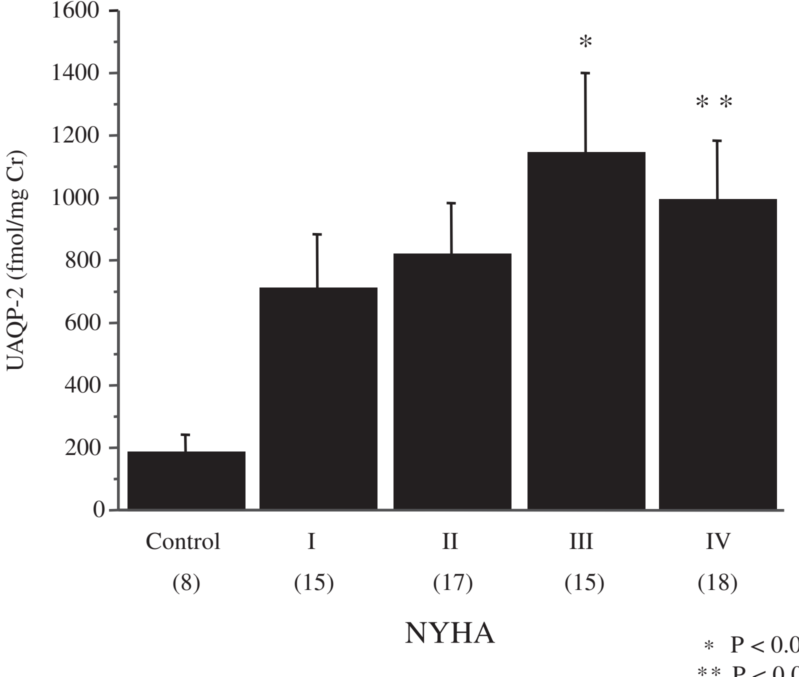

Fig. 1. Urinary excretion of AQP-2 (UAQP-2) in the patients with congestive heart failure (cited from Funayama et al., 2004).

AQP-2 was markedly increased in association with the upper classes of NYHA in the patients. Specifically, its excretion in the subgroups of NYHA III and IV were significantly greater than that in the control subjects ($p<0.02$). Urinary excretion of AQP-2 had a positive correlation with plasma AVP levels. However, it did not correlate with urinary sodium excretion and urinary osmolality. Thus, enhancement in urinary excretion of AQP-2 is derived from the augmented antidiuretic action of AVP.

AQP-2 expression in animal models

Several in vivo studies have shown the upregulation of AQP-2 mRNA and protein expression in animal models of impaired water excretion to be dependent on non-suppressible release of AVP. An increase in AQP-2 expression was demonstrated in rats with congestive heart failure (Xu et al., 1997). In the heart failure rats ligated left coronary artery, cardiac index and plasma osmolality were significantly reduced and plasma AVP increased compared to the sham-operated rats. AQP-2 mRNA and protein abundance were both significantly increased in the kidneys of the rats with chronic heart failure. The administration of OPC-31260, an AVP V_2 receptor antagonist, significantly diminished the AQP-2 mRNA and protein abundance in the rats with chronic heart failure. This in vivo study is closely related to the observation of an exaggeration of urinary excretion of AQP-2 in the patients with congestive heart failure (Funayama et al., 2004).

Patients with hypopituitarism have impaired water excretion. Particularly, dysfunction of pituitary–adrenal axis is involved in the mechanism. Recently, we demonstrated that more severe hyponatremia was found as hypopituitarism was discovered in more elderly subjects (Yatagai et al., 2003). Glucocorticoid deficiency is an animal model of hypopituitarism, and it causes impaired water excretion, secondary to the non-suppressible release of AVP (Ishikawa and Schrier, 1982). Water retention was more manifest in the aged rats with glucocorticoid deficiency than the young ones. The expression of AQP-2 mRNA and protein in kidney was increased by 1.4–1.6-fold in the glucocorticoid-deficient rats over the controls (Saito et al., 2000). Such increased expression persisted after an acute water load. In the aged rats the expression of AQP-2 mRNA and protein was significantly reduced (Preisser et al., 2000). By contrast, its expression was not reduced, but instead persisted in the aged rats with glucocorticoid deficiency (in preparation). This observation was related to non-suppressed levels of plasma AVP despite the hypoosmotic state in aged, glucocorticoid-deficient rats. Either an AVP V_2 receptor antagonist or hydrocortisone replacement abolished the augmented expression of AQP-2 mRNA and protein during glucocorticoid deficiency. There was no alteration in $[^3H]$AVP receptor binding and AVP V_2 receptor mRNA expression between the glucocorticoid-deficient and the control rats. Though plasma AVP levels remained in the normal range, chronic non-suppressible levels of plasma AVP in the hypoosmotic condition could maintain long-term stimulation on V_2 receptor-mediated cellular signalling.

The non-suppressed release of AVP frequently results in hyponatremia in SIADH. However, the degree of hyponatremia is limited by a process that counters the water-retaining action of AVP, and has called "AVP escape" phenomenon (Gross et al., 1983). We made experimental SIADH rats similar to that described in our previous reports (Fujisawa et al., 1993). SIADH rats were infused dDAVP (5 ng/h) subcutaneously by osmotic minipumps and fed the liquid diet. The other group of rats was infused dDAVP (5 ng/h) by osmotic minipumps and given commercial rat pelleted chow with free access to water (dDAVP-excess rats). In the experimental SIADH rats serum Na levels decreased to below 120 mmol/l on day 2, and hyponatremia remained less than 120 mmol/l throughout the rest of observation period. The SIADH rats failed to concentrate urine maximally. Uosm relatively increased to 1700 mmol/kg on day 2, followed by the prompt decline to approximately 800 mmol/kg (Saito et al., 2001). This alteration was reflected by an increase in body fluid because the rats were fed only by a liquid diet. However, the dDAVP-excess rats showed a distinctly different water balance from that of the

SIADH rats. There was no change in serum Na levels during the observation period. Urinary concentrating ability persisted in the dDAVP-excess rats: a decrease in urine volume and an increase in Uosm more than 3,000 mmol/kg were more manifest than those found in the SIADH rats. This may be compensated, in part, by the reduced water intake.

Figure 2 shows the expression of AQP-2 mRNA in kidneys of the rats with experimental SIADH and dDAVP-excess. There was a major transcript at 1.5 kb, which represents AQP-2 mRNA. 4.4 kb also was detected, that is, alternative splicing or polyadenylation variants. The expression of AQP-2 was promptly increased to 182.6 ± 13.6% on day 2, followed by a gradual decline during the rest of the observation period in the experimental SIADH rats. By contrast, in the dDAVP-excess rats the expression progressively increased to 323.7 ± 23.8% on day 7, without any reduction. Therefore, there was a significant difference in AQP-2 mRNA expression between the two groups of rats (Saito et al., 2001). Quite similar results were obtained for the expression of AQP-2 protein. AQP-2 protein expression was increased to a maximal level of 220.6 ± 18.2% on day 2, and it gradually declined throughout the rest of observation period in the SIADH rats. An increase in AQP-2 protein was evident in the dDAVP-excess rats.

The down-regulation of AVP V_2 receptor function was comparable in the two groups of rats. The maximal binding capacity fell to the nadir on day 2 and was thereafter suppressed at approximately 60% of the controls during the observation period.

The present study clarified that there were two reciprocal alterations in renal collecting duct of the SIADH rats: a prompt reduction in AVP V_2 receptor binding capacity and V_2 receptor mRNA expression, and up-regulation of AQP-2 mRNA expression. The down-regulation of AVP V_2 receptor was comparable in the SIADH and the dDAVP-excess rats, but the up-regulation of AQP-2 gene was significantly less in the SIADH rats than in the dDAVP-excess rats. The alteration of AQP-2 gene expression is therefore independent of cellular signal transduction of AVP. The SIADH rats had both volume expansion and hyponatremia, but the volume state appears to be the likely critical difference. A possibility is that either hypervolemia or hypotonicity attenuates the post-receptor signalling of AVP, particularly the transcriptional regulation of AQP-2 gene.

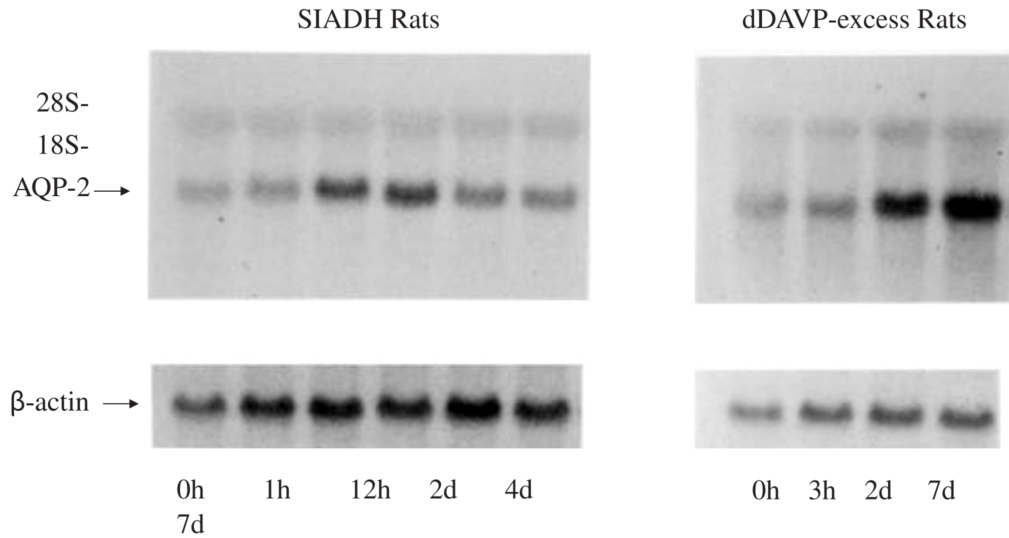

Fig. 2. Expression of AQP-2 mRNA in kidney of experimental SIADH rats and dDAVP-excess rats (cited from Saito et al., 2001).

Tonicity regulates the AQP-2 promoter

The 5′-flanking region of AQP-2 has a cAMP-response element (CRE) located at −310 to −304 bp (GACGTCA) and a tonicity-response enhancer (TonE) located at −570 to −560 bp (TGGAAATTTGT). AVP induces AQP-2 transcription by CRE, and regulates the abundance of AQP-2 protein. A recent in vitro study may suggest the presence of osmotic control of AQP-2 transcription in primary culture of inner medullary collecting duct cells (Storm et al., 2003). However, the exact mechanism of transcriptional regulation by tonicity of the AQP-2 promoter has not been determined yet.

We isolated and characterized the murine AQP-2 gene promoter region. The murine AQP-2 gene had a total of 14 kb of flanking sequence (∼10.5 kb from the 5′ side and 3.5 kb from the 3′ side of the initiation codon) into pSPORT-1 from overlapping λ clones. All primers for PCR amplications of the murine AQP-2 promoter region were constructed (Kasono et al., 2005). After cutting the PCR products with SfiI and EcoRI, the fragments were cloned into the modified pGL-3 basic vector. The 5′-flanking regions of the murine AQP-2 were prepared, and they were named −9.5AQP2, −6.1AQP2, −4.3AQP2, −2.6AQP2, −1.1AQP2 and −0.36AQP2, respectively. They were transiently transfected into Madin-Darby canine kidney (MDCK) cells or murine $IMCD_3$ cells to determine luciferase (Luc) activity.

The cDNA of human TonEBP had a large open reading frame of 1455 amino acids. This protein has a bipartite structure. A truncated TonEBP containing only the DNA-binding site (N-terminal 472 amino acids) showed dominant-negative activity on TonE-mediated stimulation of receptor gene expression. TonEBP and the truncated TonEBP cloned into pcDNA3, and named pTonEBP and pDNTonEBP, respectively, were kindly provided by Prof. H. M. Kwon.

Hypertonicity study

A 6 h exposure of cells to 0.5 μmol/l dibutyryl cAMP (DBcAMP) significantly increased the fold induction of Luc activity of −1.1AQP2 and −9.5AQP2. Hypertonicity (600 mmol/kg) significantly increased the fold induction of Luc activity of −9.5AQP2, however, the Luc activity of −1.1AQP2 containing TonE was unexpectedly not stimulated by hypertonicity. Then, we examined the effects of both hypertonicity and DBcAMP on the Luc activity. Co-stimulation of hypertonicity and DBcAMP synergistically increased the Luc activity of −9.5AQP2, but the Luc activity of −1.1AQP2 induced by DBcAMP was not significantly altered by the hypertonicity. Luc activities of several constructs (−0.36, −1.1, −2.6, −4.3, −6.1 and −9.5AQP2) of the 5′-flanking region of murine AQP-2 gene were determined under hypertonic conditions. Only the Luc activities of −6.1 and −9.5AQP2 were significantly increased, suggesting the main hypertonicity-response regions were located in −6.1 through −4.3 kb of the 5′-flanking region of the murine AQP-2 promoter. This was further confirmed by the study using pDNTonEBP. Co-transfection with −6.1AQP2 and pDNTonEBP did not affect the fold induction of the hypertonicity-induced Luc activity of −6.1AQP2. Finally, to achieve the overexpression of TonEBP we performed the transfection of −1.1AQP2 or −6.1AQP2 with pTonEBP (Fig. 3). Co-transfection with −1.1AQP2 and pTonEBP in the isotonic medium markedly augmented the Luc activity. In the same experiment the co-existence of hypertonicity and pTonEBP did not stimulate the Luc activity of −1.1AQP2. pTonEBP in the isotonic medium also increased the fold induction of Luc activity of −6.1AQP2. In this case, hypertonicity further enhanced the Luc activity of −6.1AQP2 under the overexpression of TonEBP.

The present study demonstrated that hypertonic stimulation did not significantly activate the AQP-2 promoter with <4.3 kb of the 5′-flanking region, though TonE is contained at −570 to −560 bp. There is only one TonE located in the 5′-flanking region of the AQP-2 gene up to 10.5 kb. The findings suggested the presence of an additional tonicity-responsive element in the 5′-flanking region of the AQP-2 gene located at −6.1 to −4.3 kb, in addition to TonE. The region located between −6.1 and −4.3 kb is dominantly involved

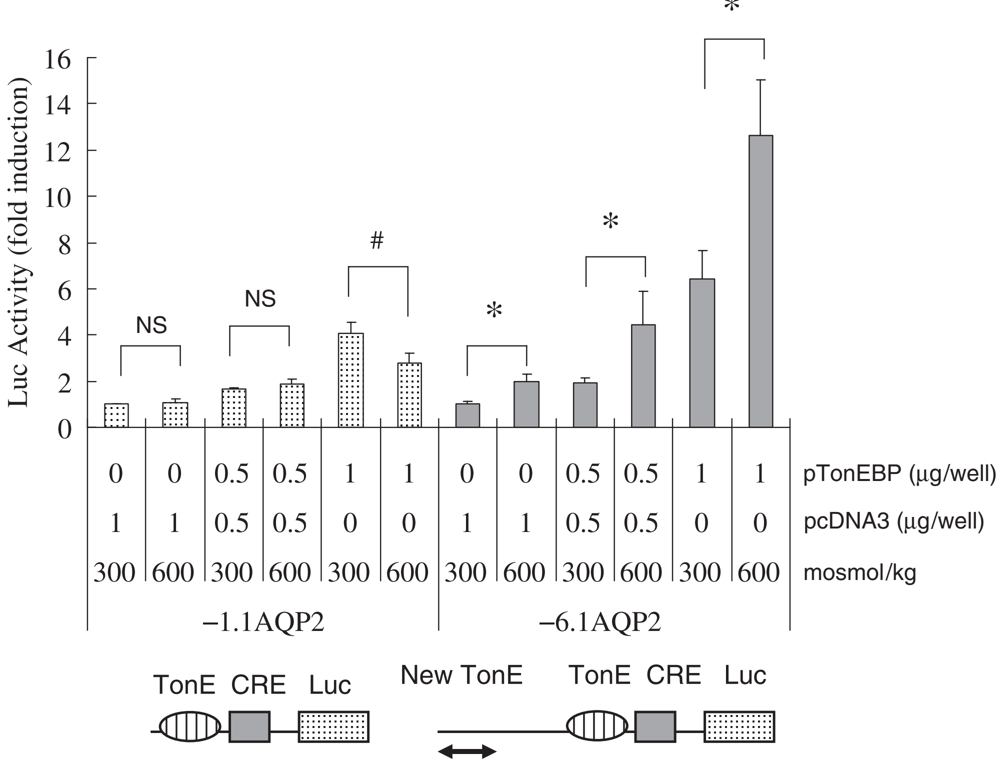

Fig. 3. Effect of hypertonicity on the Luc activity of –1.1AQP2 and –6.1AQP2 under the over-expression of TonEBP (cited from Kasono et al., 2005).

in hypertonicity-induced transcription of AQP-2 promoter, and it is necessary to cooperate with classical TonE to promote its transcriptional regulation (Kasono et al., 2005). However, Hasler et al. (2006) reported in mouse collecting duct principal cells TonEBP directly acts on the AQP-2 transcriptional activity and hypertonic challenge increases AQP-2 promoter activity mediated via TonEBP.

Hypotonicity study

Next, we determined whether hypotonicity regulates the transcription of AQP-2 using −9.5AQP2, −6.1AQP2, −1.1AQP2 and −0.36AQP2. They were transfected with the vector for AQP-2 cDNA into mIMCD$_3$ cells, and Luc activity was measured in response to 5 µmol/l DBcAMP under hypotonic condition (225 mmol/kg). Figure 4 shows the effect of hypotonicity on DBcAMP-induced Luc activity in the cells co-transfected with −1.1AQP2 and murine AQP-2. The Luc activity in response to 5 µmol/l DBcAMP significantly increased in a time-dependent manner in the isotonic condition. In the hypotonic medium such response to DBcAMP was totally blunted. Similar results were obtained with the cells transfected with −9.5AQP2 and −6.1AQP2. In contrast, when the study was carried out with −0.36AQP2, 5 µmol/l DBcAMP stimulates the Luc activity in both the isotonic and hypotonic medium. Moreover, hypotonic medium did not affect the increase in DBcAMP-induced Luc activity of −1.1AQP2 when the cells were cotransfected with pDNTonEBP. These findings may indicate that inhibitory effect of hypotonic condition is probably mediated via TonE itself.

1 µmol/l SP600125, an inhibitor of JNK kinase, totally restored the response of Luc activity in the cells co-transfected with −1.1AQP2 and murine AQP-2 in the hypotonic medium. However,

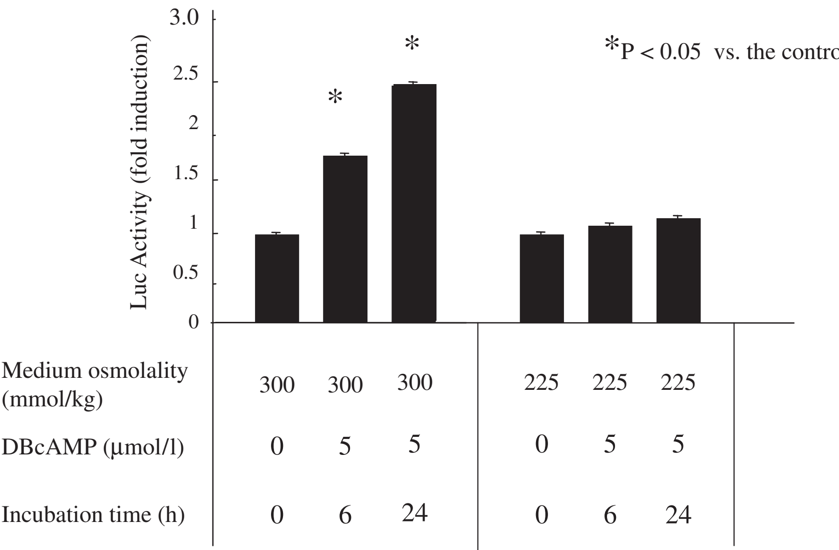

Fig. 4. Effect of hypotonicity on the AQP-2 promoter activity when the cells were co-transfected with –1.1AQP2 and murine AQP-2.

neither 1 μmol/l SB203580 (p38 MAP kinase inhibitor) nor U0126 (MEK inhibitor) affected the Luc activity of −1.1AQP2. Thus, JNK could be involved in transduction of hypotonic signal to the tonicity response element, producing an inhibition of CRE. It is known that p38MAP kinase and MEK facilitate hypertonic regulation of AQP-1 promoter via TonE (Umenishi and Schrier, 2003). These findings therefore implicate that there are different intracellular signals regulating AQP-2 promoter gene between hypo- and hypertonicity.

Whether osmolality or tonicity is important for the Luc activity of AQP-2 promoter gene was examined. In the isoosmotic, but actually hypotonic medium (approximately 225 mmol/kg) which was made by adding excess urea to the hypotonic medium, basal Luc activity remained unchanged, but the response to 5 μmol/l DBcAMP was totally blunted. A finding was quite similar to that in the cells exposed to the hypotonic medium of 225 mmol/kg, as noted earlier. As urea can move between intracellular and extracellular space across cell membrane, these findings may implicate that osmotic gradient between outside and inside of cells affects the Luc activity of AQP-2 promoter.

As described earlier, there is ‘‘AVP escape’’ phenomenon from antidiuresis under chronic AVP excess condition. We suggested that hypotonicity might diminish the post-receptor signalling of AVP in collecting duct in the experimental SIADH rats (Saito et al., 2001). The present in vitro study revealed the direct involvement of hypotonicity in reducing AQP-2 promoter activity. Reduction in transcriptional regulation of AQP-2 under hypotonic state may support the in vivo finding of AVP escape phenomenon in chronic AVP-induced antidiuresis.

Acknowledgments

The study was supported by the grants from The Ministry of Education, Science and Culture of Japan (Grant nos. 13671160, 13137208 and 17590841).

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I.D. Neumann and R. Landgraf (Eds.)
Progress in Brain Research, Vol. 170
ISSN 0079-6123
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CHAPTER 46

Potential utility of aquaporin modulators for therapy of brain disorders

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Abstract: Of the several aquaporin (AQP) water channels expressed in the central nervous system, AQP4 is an attractive target for drug discovery. AQP4 is expressed in astroglia, most strongly at the blood–brain and brain–cerebrospinal fluid barriers. Phenotype analysis of AQP4 knockout mice indicates the involvement of AQP4 in three distinct processes: brain water balance, astroglial cell migration and neural signal transduction. By slowing water uptake into the brain, AQP4 knockout mice manifest reduced brain swelling and improved outcome in models of cytotoxic cerebral oedema such as water intoxication, focal ischaemia and meningitis. However, by slowing the clearance of excess water from brain, AQP4 knockout mice do worse in models of vasogenic oedema such as brain tumour, abscess and hydrocephalus. AQP4 deficient astroglial cells show greatly impaired migration in response to chemotactic stimuli, reducing glial scar formation, by a mechanism that we propose involves AQP4-facilitated water flux in lamellipodia of migrating cells. AQP4 knockout mice also manifest increased seizure threshold and duration, by a mechanism that may involve slowed K^+ uptake from the extracellular space (ECS) following neuroexcitation, as well as ECS expansion. Notwithstanding challenges in drug delivery to the central nervous system and their multiplicity of actions, AQP4 inhibitors have potential utility in reducing cytotoxic brain swelling, increasing seizure threshold and reducing glial scar formation; enhancers of AQP4 expression have potential utility in reducing vasogenic brain swelling. AQP4 modulators may thus offer new therapeutic options for stroke, tumour, infection, hydrocephalus, epilepsy and traumatic brain and spinal cord injury.

Keywords: AQP4; water transport; transgenic mouse; brain oedema; cell migration; epilepsy

Introduction

Excess accumulation of brain water occurs in a wide range of brain disorders such as stroke, tumour, infection, hydrocephalus and traumatic injury. Brain oedema results in elevated intracranial pressure, potentially leading to brain ischaemia, herniation and death. Current treatments for brain oedema, which include hyperosmolar agents and surgical decompression, have changed little since their introduction more than 80 years ago. As described in this review, the water transporting protein aquaporin-4 (AQP4) provides an important route for water movement between blood and brain, and between brain and CSF compartments. Distinct from its involvement in transcellular water

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DOI: 10.1016/S0079-6123(08)00446-9

transport and brain swelling, AQP4 involvement in astroglial cell migration and neural signal transduction has been discovered. Each of these distinct roles of AQP4 suggests specific therapeutic strategies, such as AQP4 inhibition to reduce brain oedema and seizure susceptibility, and enhance neuronal regeneration following brain injury. This review assesses experimental evidence in support of the potential indications of AQP modulators in brain therapy, and discusses challenges in their implementation. Our focus will be on AQP4, because of its potential in translational developments, with brief discussion of two other AQPs, AQP1 and AQP9, that are expressed in brain.

Aquaporins in normal brain

AQP4, the principal brain water channel, is expressed in glia at the borders between major water compartments and brain parenchyma (Nielsen et al., 1997; Rash et al., 1998) including astroglial foot processes (blood–brain barrier), glia limiting membrane (subarachnoid cerebrospinal fluid–brain interface) as well as ependyma and subependymal astroglia (ventricular cerebrospinal fluid–brain interface). This pattern of expression suggests that AQP4 controls the flow of water into and out of the brain, and may thus play a key role in brain oedema. Two AQP4 splice variants are expressed in brain, termed M1 and M23, which can form homo- and heterotetramers, respectively, which in turn assemble into square lattices of tetramers within astroglial cell plasma membranes (Yang et al., 1996; Verbavatz et al., 1997; Furman et al., 2003; Rash et al., 2004; Silberstein et al., 2004). The relative abundance of M1 vs. M23 isoforms determines the size of orthogonal arrays, which can contain up to several hundred tetramers (Furman et al., 2003), and possibly influences AQP4 single channel water permeability (Silberstein et al., 2004). Compared with non-interacting AQP4 tetramers, orthogonally inter-connected tetramers are proposed to allow more efficient anchoring of AQP4 to intracellular proteins such as alpha syntrophin. For example, for polarization of AQP4 expression in astroglial endfeet each non-interacting AQP4 tetramer would require its own intracellular anchor, whereas an orthogonal arrangement might require only one anchor per raft.

The other major brain water channel, AQP1, is found primarily in the ventricular-facing (apical) membrane of choroid plexus epithelium, where it plays a role in the formation of cerebrospinal fluid (Oshio et al., 2005). Interestingly, AQP1 is not expressed in normal brain vascular endothelial cells, although it is ubiquitously present in peripheral vascular endothelia (Nielsen et al., 1993; Dolman et al., 2005). The observations that brain endothelial cells cultured in the absence of astroglia express AQP1, as do brain tumour endothelia (which are not surrounded by glia), suggest that astroglia produce signals to inhibit AQP1 expression in the brain vascular endothelium.

It has been suggested that AQP9 is also expressed in the brain (Badaut and Regli, 2004). AQP9 immunoreactivity was detected in a subpopulation of glia called tanycytes, in some astroglia, and in glucose-sensitive catecholaminergic neurons. Because AQP9 also transports small molecules, including glycerol and lactate, it has been suggested that AQP9 may participate in brain energy metabolism (Badaut and Regli, 2004). AQP9 expression in mitochondria (Amiry-Moghaddam et al., 2005) provides further evidence for the involvement of AQP9 in energy metabolism. However, recent studies have questioned the presence and physiological role of AQP9 in brain. A major problem with AQP9 research is the lack of good anti-AQP9 antibodies for immunohistochemical experiments, as evidenced by the comparable AQP9 immunoreactivity detected in the brains of wildtype and AQP9 null mice (Rojek et al., 2007). Also, recent functional studies and theoretical arguments argue against water channel expression and function in mitochondria (Yang et al., 2006b).

Aquaporin regulation studies

Although the pattern of water channel expression in the brain provides important clues about AQP function, further information has been obtained

from regulation studies. In general, AQP4 expression becomes upregulated in brain astroglia after injury including trauma, tumour and haemorrhage (Papadopoulos and Verkman, 2007). In human astrocytomas, AQP4 expression in tumours correlates with the presence of brain swelling on magnetic resonance scans (Saadoun et al., 2002), raising the possibility that AQP4 participates in the formation and/or absorption of brain tumour oedema. Interestingly, dexamethasone, which is commonly used clinically to reduce brain tumour oedema, does not alter AQP4 expression, with the AQP4 promoter lacking steroid response elements. Therefore, AQP4 modulators may act synergistically with corticosteroids in reducing brain tumour oedema. Increased expression of AQP4 in reactive and malignant astroglia suggests that AQP4 may play further roles in the functions of these cells, such as in glial scar formation and the growth/spread of astroglial tumours. AQP1, which is normally found in the choroid plexus, becomes upregulated in choroid plexus tumours, which are associated with increased cerebrospinal fluid production (Longatti et al., 2006). There is also evidence that AQP9 immunoreactivity changes in response to injury (Badaut and Regli, 2004), though as mentioned above there are significant concerns about the specificity of anti-AQP9 antibodies. In general, regulation studies have provided indirect evidence about the functions of AQPs in the brain. Most of our knowledge about AQP functions comes from studies using AQP null mice.

Roles of aquaporins in brain

AQPs have three major functions in the brain: control of brain water balance, cell migration and neuronal excitability (summarized in Fig. 1). The movement of water into and out of the brain parenchyma is primarily controlled by AQP4, with AQP1 contributing to the formation of cerebrospinal fluid by the choroid plexus. AQP4 facilitates the migration of reactive astroglia towards a site of injury, and may also accelerate the migration of malignant astroglia. AQP1 facilitates the migration of endothelial cells, thus accelerating angiogenesis in growing tumours. AQP4 controls the size of the extracellular space (ECS) and extracellular K^+ kinetics, which are important components of neuronal excitability. These three major AQP functions are discussed in more detail below.

Brain water balance

The pattern of AQP4 expression in the brain (at the borders between brain parenchyma and major fluid compartments) as well as regulation studies (correlating AQP4 expression and brain oedema), provide indirect evidence for involvement of AQP4 in brain water balance. Table 1 summarizes evidence supporting involvement of AQP4 in brain water balance. Direct evidence came from experiments showing reduced brain swelling and improved survival in AQP4 null versus wildtype mice after water intoxication (Fig. 2A), reduced hemispheric swelling after focal cerebral ischaemia (Fig. 2B) and improved survival after bacterial meningitis (Fig. 2C) (Manley et al., 2000; Papadopoulos and Verkman, 2005). Reduced brain swelling was also reported in alpha-syntrophin null mice, which secondarily have disrupted brain AQP4 expression (Amiry-Moghaddam et al., 2003a, 2004). According to the Klatzo classification of brain oedema (Klatzo, 1994), these are primarily models of cytotoxic (cell swelling) oedema in which excess water moves from the vasculature into the brain parenchyma through an intact blood–brain barrier (Fig. 1A). The forces driving water flow to form cytotoxic oedema are osmotic, generated in water intoxication by reduced plasma osmolality and in ischaemia by failure of the Na^+/K^+ ATPase with consequent Na^+ and water flow from the intravascular and extracellular compartments into the intracellular compartment. Taken together, these studies suggest that by reducing the flow of water from the vasculature into the brain parenchyma, AQP4 limits the rate of brain water accumulation in cytotoxic oedema. Therefore, AQP4 inhibitors or downregulators are predicted to be powerful anti-cytotoxic oedema agents.

Subsequent experiments showed that AQP4 also plays a key role in elimination of excess brain

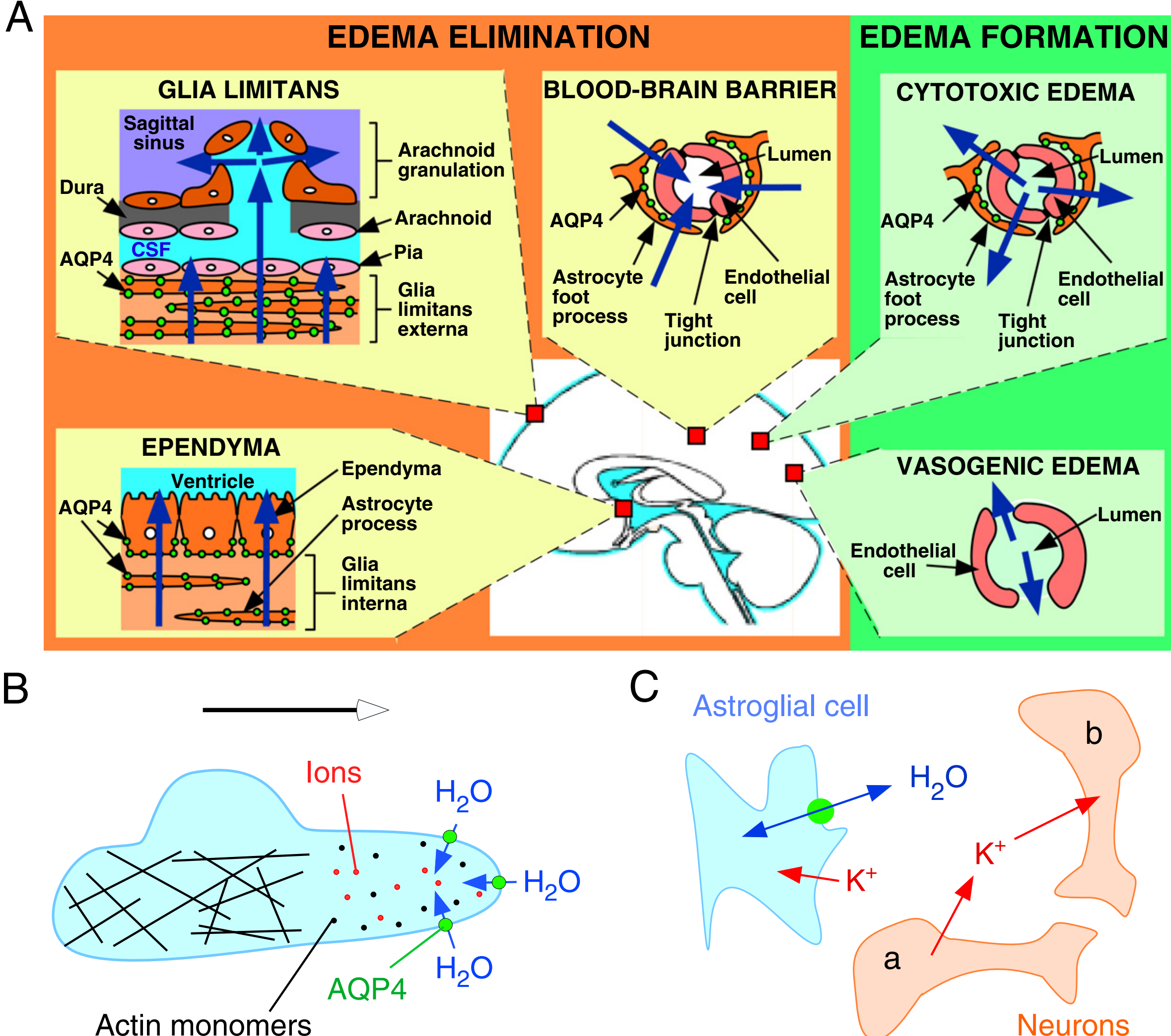

Fig. 1. Schematic depicting three distinct roles of AQP4 (green circles) in brain function: (A) brain water balance, (B) astroglial cell migration and (C) neuronal excitation. (A) Green. Routes of oedema formation in the two types of brain oedema (cytotoxic — through AQP4, vasogenic — through interendothelial spaces). Orange. Oedema fluid is eliminated by AQP4 through the glial limitans into subarachnoid CSF, through ependyma and sub-ependymal astroglia into ventricular CSF, and through astroglial pericapillary foot processes into blood. (B) AQP4 polarizes to the leading edge of migrating astroglia and accelerates cell migration. AQP4 facilitates water entry into lamellipodial protrusions in response to intracellular hyperosmolality produced by actin depolymerization and ion influx. (C) AQP4 deletion reduces neuroexcitation. Active neurons (neuron a) release K^+ into the extracellular space (ECS). Increased extracellular $[K^+]$ depolarizes quiescent neurons (neuron b). AQP4 deletion increases ECS volume and reduces astroglial cell K^+ reuptake. This buffers the increase in extracellular $[K^+]$ by active neuron a, preventing depolarization of quiescent neuron b. See text for further explanations. (See Color Plate 46.1 in color plate section.)

water. When the blood–brain barrier becomes disrupted (brain tumour, brain abscess, focal freeze injury), water moves from the vasculature into the ECS of the brain in an AQP4-independent manner down a hydrostatic gradient to form, what was termed by Klatzo (1994), vasogenic oedema. Excess water is eliminated primarily through the glia limiting membrane into the cerebrospinal fluid

Table 1. Summary of studies implicating involvement of AQP4 in brain oedema

Organism	Evidence	Principal finding	Pathological process	References
Human	Indirect	Increased AQP4 expression correlates with oedema	Astrocytoma	Saadoun et al., 2002; Warth et al., 2007
Human	Indirect	Increased AQP4 expression	Brain tumours Subarachnoid haemorrhage	Badaut et al., 2003
Human	Indirect	Increased AQP4 expression	Cerebral infarct	Aoki et al., 2003
Rat	Indirect	Changes in AQP4 expression correlate with oedema	Cerebral ischaemia	Meng et al., 2004; Badaut et al., 2007; Chen et al., 2007
Mouse	Indirect	Changes in AQP4 expression correlate with oedema	Cerebral ischaemia	Ribeiro Mde et al., 2006
Rat	Indirect	Changes in AQP4 expression correlate with oedema	Traumatic brain injury	Kiening et al., 2002; Sun et al., 2003; Chen et al., 2007
Rat	Indirect	Increased AQP4 immunoreactivity	Hyponatremia	Ke et al., 2002
Rat	Indirect	High AQP4 expression correlates with BBB opening	Median forebrain bundle injury	Vizuete et al., 1999
Mouse	Direct	Reduced brain oedema in AQP4 null mice	Water intoxication, cerebral ischaemia	Manley et al., 2000
Mouse	Direct	Reduced brain oedema in alpha syntrophin null mice	Water intoxication, cerebral ischaemia	Amiry-Moghaddam et al., 2003a, 2004
Mouse	Direct	Reduced brain oedema in AQP4 null mice	Bacterial meningitis	Papadopoulos and Verkman, 2005
Mouse	Direct	Increased brain oedema in AQP4 null mice	Brain melanoma, focal cortical freeze, aCSF infusion	Papadopoulos et al., 2004
Mouse	Direct	Increased brain oedema in AQP4 null mice	Brain abscess	Bloch et al., 2005
Mouse	Direct	Increased hydrocephalus in AQP4 null mice	Hydrocephalus	Bloch et al., 2006

(Klatzo, 1994). There is increased brain water gain and intracranial pressure in AQP4 null versus wildtype mice with brain tumour, brain abscess, focal cortical freeze injury and after infusion of normal saline directly into brain ECS (Fig. 2D) (Papadopoulos et al., 2004; Bloch et al., 2005), suggesting that vasogenic oedema fluid is eliminated by an AQP4-dependent route (Fig. 1A). Therefore, AQP4 activators or upregulators are predicted reduce brain swelling in pathologies associated with vasogenic brain oedema. Kaolin injection into the cisternal magna obstructs cerebrospinal fluid outflow from the 4th ventricle (Bloch et al., 2006). AQP4 null mice develop more marked hydrocephalus than wildtype mice after kaolin injection, probably due to reduced transependymal water clearance in the AQP4 null mice (Fig. 2E). AQP4 inhibitors may thus provide primary or adjunctive medical therapy in obstructive hydrocephalus.

AQP1 controls water movement from blood vessels into the ventricles in the formation of cerebrospinal fluid. Compared with wildtype mice, in AQP1 null mice the rate of cerebrospinal fluid formation is reduced by about 25%, which was associated with reduced osmotic permeability of the choroid plexus epithelium and a twofold decrease in intracranial pressure (Oshio et al., 2005). Though these changes are modest, and in part related to reduced central venous pressure in AQP1 null mice, it was suggested that AQP1 inhibitors might be useful in the treatment of hydrocephalus or benign intracranial hypertension (pseudotumour cerebri).

Astroglial cell migration

After brain injury, astroglia throughout the brain become reactive and migrate towards the lesion site to form a glial scar (Kastner, 1987; Fawcett

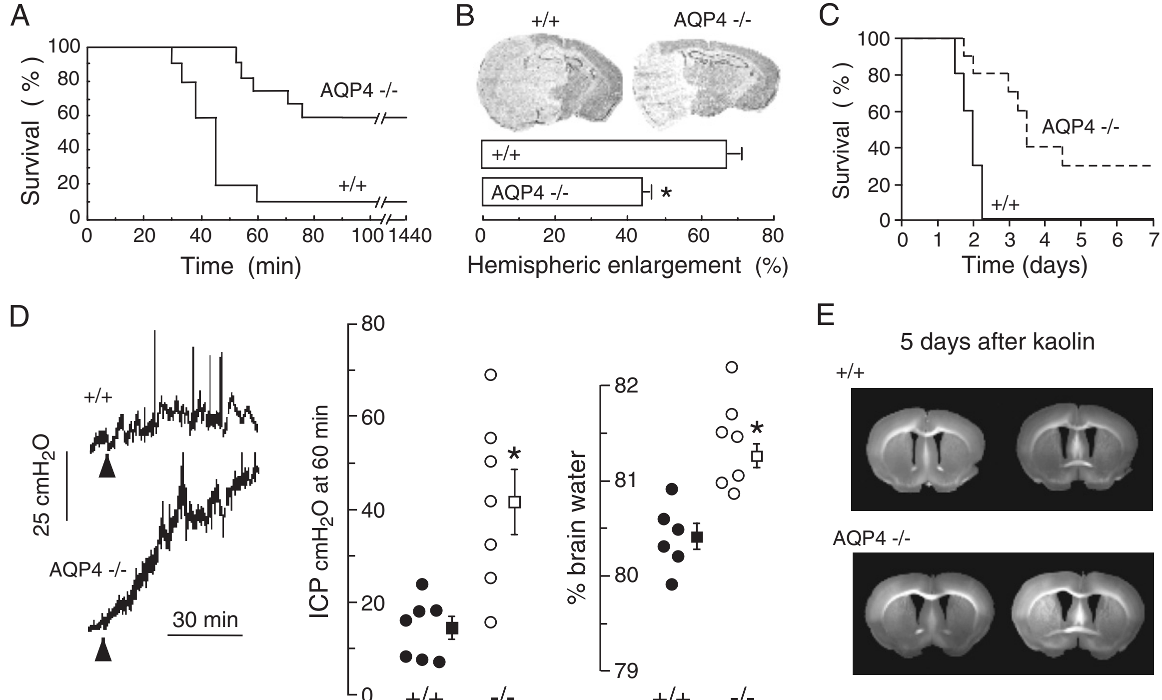

Fig. 2. AQP4 deletion reduces brain water accumulation in cytotoxic oedema, but slows removal of excess brain water in vasogenic oedema. (A) Water intoxication model of cytotoxic oedema. Survival of 12 wildtype vs. 12 AQP4 knockout mice after acute water intoxication produced by intraperitoneal water injection (20% body weight). (B) (Top) Ischaemic stroke model of cytotoxic oedema. Brain sections of mice at 24 h after ischaemic stroke produced by permanent middle cerebral artery occlusion. (Bottom) Average hemispheric enlargement expressed as a percentage determined by image analysis of brain sections (7 $AQP4^{+/+}$ vs. 7 $AQP4^{-/-}$ mice, SEM, $^{*}P<0.0002$). (C) Mouse survival (10 $AQP4^{+/+}$ vs. 10 $AQP4^{-/-}$ mice, $P<0.001$) in a bacterial model of meningitis produced by cisternal injection of *S. pneumoniae*. (D) Reduced elevation in intracranial pressure (ICP, SEM, $^{*}P<0.01$) and brain water content (SEM, $^{*}P<0.001$) following continuous intraparenchymal infusion of artificial cerebrospinal fluid at 0.5 μL/min. (E) Accelerated progression of hydrocephalus in AQP4 null mice. Coronal sections wildtype and AQP4 null mouse brain at 5 days after kaolin injection. Data from Manley et al., 2000, Papadopoulos et al., 2004 and Bloch et al., 2006.

and Asher, 1999). Recent evidence suggests that AQP4, which becomes over-expressed in reactive astroglia, facilitates astroglial cell migration (Saadoun et al., 2005b; Auguste et al., 2007). Reduced migration speed was seen in cultured AQP4 null versus wildtype astroglia in transwell (Fig. 3A) and in vitro scratch assays, and was associated with reduced glial scarring in AQP4 null mice after a cortical stab injury. Further studies showed that increased cell migration in AQP expressing cells is a general phenomenon independent of AQP type and cell type (Saadoun et al., 2005a; Hara-Chikuma and Verkman, 2006; Hu and Verkman, 2006; Levin and Verkman, 2006; Loitto et al., 2007). AQP1 facilitates migration in endothelial cells, Chinese Hamster Ovary, Fisher Rat Thyroid, renal proximal tubule cells, as well as malignant melanoma and breast carcinoma cells in vitro and in vivo. AQP3 facilitates migration in skin keratinocytes and corneal epithelial cells. Two possible mechanisms involved in AQP-dependent cell migration have been proposed (Papadopoulos et al., 2008). According to the first hypothesis, AQPs may facilitate the movement of water across the cell membrane at the leading edge of the cell in response to rapid changes in intracellular osmolality that occur in this region. This mechanism is consistent with the increased number of cell

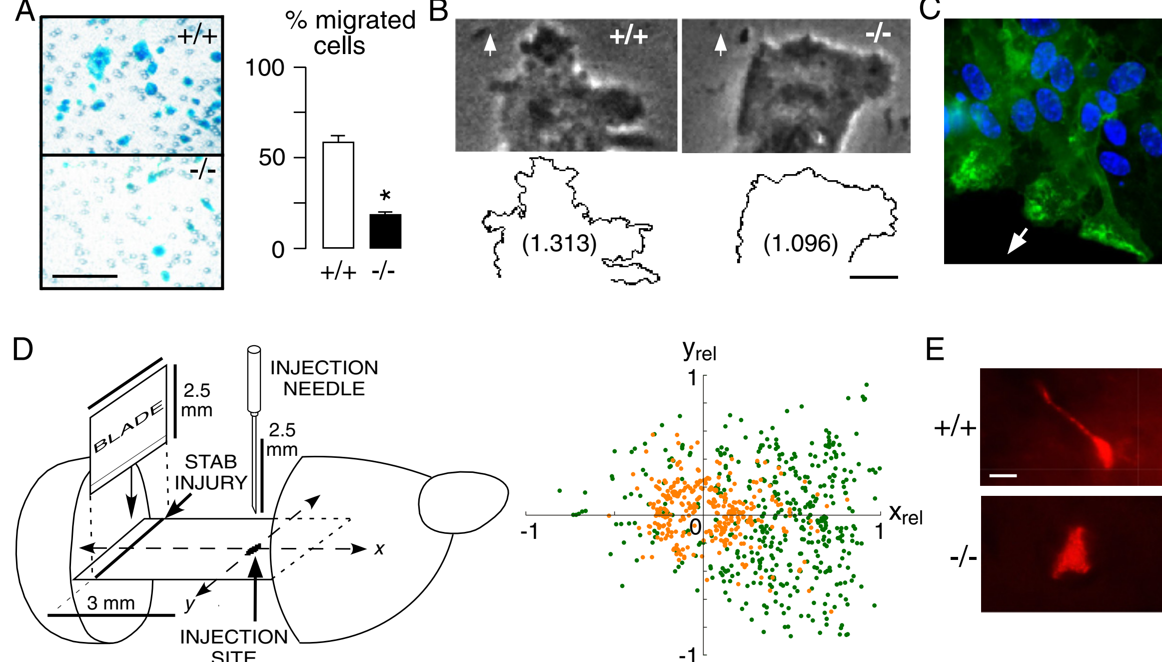

Fig. 3. AQP4 facilitates astroglial migration in vitro and in brain. (A) Left. Boyden chamber migration assay showing AQP4$^{+/+}$ and AQP4$^{-/-}$ astroglia (blue) after scraping off the non-migrated cells. Astroglia were plated on the top chamber of porous transwell filter (2.8×10^4/cm^2) and were allowed to migrate for 6 h towards 10% FBS as chemoattractant. Bar, 100 μm. Right. Summary of migration experiments (SEM, $^*P<0.001$). (B) Phase contrast micrographs (Top) and outline (Bottom) of the leading end of a migrating AQP4$^{+/+}$ and AQP4$^{-/-}$ astroglial cell in the in vitro wound assay. Arrows show direction of migration. Numbers are fractal dimensions (larger number denotes more irregular cell membrane). Bar 10 μm. (C) AQP4 protein (green) polarization to the front end of migrating astroglia in a wound assay. Arrow shows direction of migration. (D) Left. Stab injury/cell injection model of astroglial migration in mouse brain. Two days before cell injection, a stab was created as shown. Cultured AQP4$^{+/+}$ and AQP4$^{+/+}$ astroglia were fluorescently labelled and injected as indicated. Right. Locations of migrating fluorescently stained AQP4$^{+/+}$ (green) and AQP4$^{-/-}$ (orange) astroglia. The x-axis is relative distance between injection and stab injury sites (x_{rel}). (E) High magnification fluorescence micrographs of migrating AQP4$^{+/+}$ and AQP4$^{-/-}$ astroglia. Bar 5 μm. Data from Saadoun et al., 2005b and Auguste et al., 2007. (See Color Plate 46.3 in color plate section.)

membrane protrusions in lamellipodia of AQP expressing vs. non-expressing cells (Fig. 3B), as well as the polarization of AQPs to the front end of migrating cells (Fig. 3C) (Saadoun et al., 2005a, b). According to the second hypothesis, AQPs facilitate rapid changes in cell shape that are required for migrating cells to squeeze through the irregular ECS and to push apart stationary cells in their way. This mechanism, which would be undetectable in standard 2-dimensional migration assays, may explain why differences in migration between wildtype and AQP4 null astroglia are more marked in brain in vivo than in culture. Figure 3D summarizes an in vivo migration study of astroglia towards a stab wound in brain, which showed faster and more directional migration in wildtype vs. AQP4 null astroglia. Consistent with a major role for AQP4 in rapid cell shape changes during migration, more elongated wildtype vs. AQP4 null astroglia were seen as they migrated in intact brain (Fig. 3E). Whatever the mechanism, the ability of AQPs to accelerate cell migration has important therapeutic implications. AQP4 inhibitors may reduce glial scarring, which is the major impediment to neuronal regeneration after injury in the central nervous system (Fawcett and

Asher, 1999). Inhibition of AQP4 (and AQP1) may reduce tumour infiltration, for example the infiltration of malignant glioma cells into the surrounding brain. By reducing endothelial cell migration, AQP1 inhibitors are predicted to inhibit angiogenesis, thus reducing tumour growth.

Neural signal transduction

Phenotype analysis of AQP4 null mice has provided evidence for an unexpected role of AQP4 in neural signal transduction. AQP4 is expressed in supportive cells adjacent to electrically excitable cells, as in glia versus neurons in brain and spinal cord, Müller versus bipolar cells in retina and hair versus supportive cells in the inner ear. Based on this expression pattern, we found evidence for impaired auditory and visual signal transduction in AQP4 null mice, seen as increased auditory brainstem response thresholds (Li and Verkman, 2001) and reduced electroretinographic potentials (Li et al., 2002). In brain, seizure susceptibility in response to the convulsant (GABA antagonist) pentylenetetrazol was remarkably increased in AQP4 null mice (Binder et al., 2004a); at 40 mg/kg pentylenetetrazol, all wildtype mice exhibited seizure activity, whereas 6 out of 7 AQP4 null mice did not exhibit seizure activity. In a definitive study involving EEG recordings in freely moving mice (Fig. 4A, top), electrically induced seizures following hippocampal stimulation showed greater threshold and remarkably longer seizure duration in AQP4 null mice compared to wildtype mice (Fig. 4A, bottom) (Binder et al., 2006). In a related study, using a hyperthermia model of seizure induction, alpha syntrophin deficient mice (manifesting AQP4 mislocalization) developed more severe seizures than wildtype mice (Amiry-Moghaddam et al., 2003b).

Several lines of evidence suggest delayed K^+ uptake from brain ECS in AQP4 deficiency, which may account for the prolonged seizure phenotype. Direct measurements of K^+ concentration in brain cortex in living mice using K^+-sensitive microelectrodes showed significant slowing of K^+ clearance following local stimulation by brief electrical pulses (Fig. 4B). Using a new triazacryptand-based K^+-sensitive fluorescent dye, TAC-Red, altered K^+ wave dynamics were found in a cortical spreading depression model of neuroexcitation, again with delayed K^+ clearance (Fig. 4C). In related work, hippocampal slices from alpha syntrophin deficient mice showed slowed K^+ clearance following evoked neuronal activity (Amiry-Moghaddam et al., 2003b). Impaired K^+ clearance in AQP4 deficiency following neuroexcitation could account in part for the prolonged seizure activity in AQP4 deficiency, as well as altered evoked potential responses, but probably not for the reduced seizure susceptibility in AQP4 deficiency.

The molecular mechanism(s) responsible for impaired ECS K^+ clearance in AQP4 deficiency remain unclear. It has been proposed that AQP4 is closely associated with the inwardly rectifying K^+ channel Kir4.1. Immunocolocalization and immunopercipitation studies have suggested a close physical association of these two proteins in astroglia and Müller cells. However, recent patch-clamp studies at whole-cell and single-channel levels in astroglia (Zhang and Verkman, 2008) and Müller cells (Ruiz-Ederra et al., 2007), provide direct evidence against functionally significant interactions between AQP4 and Kir4.1. Whether AQP4-facilitated water transport alone could account for the delay in ECS K^+ clearance is unclear, as is whether AQP4 deficiency is associated with altered expression of other key proteins involved in ECS K^+ handling.

We have obtained evidence for ECS expansion in AQP4 deficiency, which may account in part for reduced seizure susceptibility and prolonged seizure duration in AQP4 deficiency. An expanded ECS would provide a larger aqueous volume to dilute K^+ released into the ECS during neuroexcitation, thereby slowing changes in ECS K^+ concentration. Evidence for an expanded ECS in AQP4 deficiency came from cortical surface photobleaching measurements of the diffusion of fluorescently labelled macromolecules (Binder et al., 2004b). In this method the ECS in mouse brain was fluorescently stained by exposure of the intact dura to fluorescein–dextran after craniectomy (Fig. 4D, left). Fluorescein–dextran diffusion was detected by fluorescence recovery

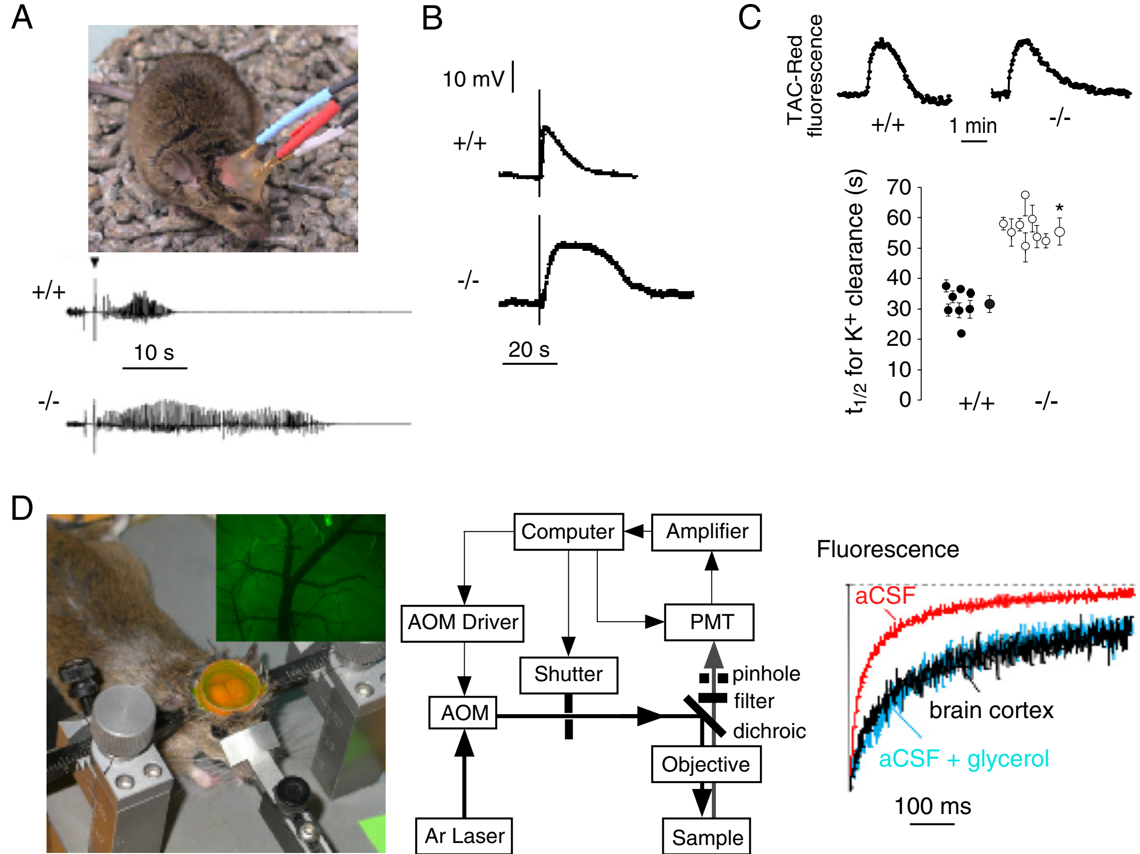

Fig. 4. AQP4 involvement in brain neuroexcitation. (A) Increased seizure duration in AQP4 null mice. Top. Bipolar electrodes implanted in the right hippocampus were connected to a stimulator and electroencephalograph recording system. Bottom. Representative electroencephalograms in freely moving mice following electrically induced generalized seizures. (B) Delayed K^+ clearance in brain following electrically induced seizure-like neuroexcitation. Measurements done using K^+-sensitive microelectrodes inserted into brain cortex in living mice. (C) Slowed K^+ clearance in brain ECS during cortical spreading depression measured using TAC-Red, a K^+-sensitive fluorescent probe. Top. Representative data. Bottom. Half-times ($t_{1/2}$) for K^+ reuptake. (D) Expanded brain ECS in AQP4 null mice measured by cortical surface photobleaching. Left. Mouse brain surface exposed to FITC-dextran with dura intact following craniectomy and fluorescence imaging of cortical surface after loading with FITC-dextran (inset). Middle. Photobleaching apparatus. A laser beam is modulated by an acousto-optic modulator and directed onto the surface of the cortex using a dichroic mirror and objective lens. Right. In vivo fluorescence recovery in cortex of wildtype mouse shown in comparison to aCSF and 30% glycerol in aCSF. Taken from Binder et al., 2006, Binder et al., 2004b and Padmawar et al., 2005. (See Color Plate 46.4 in color plate section.)

after laser-induced cortical photobleaching using confocal optics, as shown in Fig. 4D (middle). FITC–dextran diffusion was slowed ~threefold in brain ECS relative to saline solutions (Fig. 4D, right). Diffusion of FITC–dextrans was significantly accelerated in AQP4 null mice, indicating an expanded ECS. ECS expansion in AQP4 deficiency was confirmed in follow-up studies utilizing a microfiberoptic epifluorescence photobleaching method to measure diffusion in deep brain structures (Zador et al., 2008). It remains unclear, however, whether ECS expansion in AQP4 deficiency could account quantitatively for the altered ECS K^+ dynamics, as well as the mechanisms involved in chronic ECS expansion in AQP4 deficiency.

Indications and status of aquaporin modulators

Proposed indications of AQP4 modulators

Based on the above discussions, AQP4 modulators have therapeutic potential in several pathologies. The central role of AQP4 in controlling brain fluid balance suggests the utility of AQP4 modulators in the treatment of brain swelling. These drugs are expected to reduce brain water content and intracranial pressure, thus improving cerebral perfusion and preventing brain herniation and death. In this context, AQP4 modulators would be expected to act acutely and be administered for a few days whilst brain oedema remains a problem. AQP4 inhibitors or downregulators would be used to treat cytotoxic brain oedema caused by hypoxia, ischaemia or meningitis. AQP4 activators or upregulators would be useful in treating vasogenic brain oedema associated with brain tumour and brain abscess. Because the commonly used anti-oedema medications such as cortocosteroids do not modulate AQP4 function, AQP4 modulators used in combination with these drugs might have synergistic actions. AQP4 modulators may have side effects, however, potentially including auditory or visual disturbance. Such effects, if they occur, are expected to be transient and are clinically acceptable given the high morbidity and death associated with brain oedema. Many patients to be treated with AQP4 modulators will probably be comatose in Intensive Care Units, and therefore such side effects would be largely asymptomatic. AQP4 inhibitors might also be used to delay glial scar formation after brain injury to accelerate neuronal regeneration including axonal sprouting and synaptogenesis. In this context AQP4 inhibitors would be started a few days after the injury and administered for a few weeks, during the period of maximal glial scar formation. If AQP4 turns out to play an important role in the infiltration of astrocytomas, then AQP4 inhibitors might be used to treat these highly malignant tumours. By rendering astrocytomas less infiltrative with defined tumour–brain margins, AQP4 inhibitors may permit more complete surgical excision. Finally, AQP4 inhibitors may also be used to increase seizure threshold. Because their mechanism-of-action does not overlap with that of other anticonvulsants, AQP4 inhibitors might be used in combination with currently used antiepileptics.

Status and prospects for development of AQP4 inhibitors

There are at present no reported AQP inhibitors that are suitable candidates for clinical development. Though several AQPs are inhibited by sulfhydryl-reactive mercurials such as mercury and gold (Niemietz and Tyerman, 2002), these metal ions are non-specific in their action and toxic to living cells. Some candidate blockers of AQP1 have been reported, including tetraethylammonium (Brooks et al., 2000), acetazolamide (Ma et al., 2004) and DMSO (van Hoek et al., 1990); however, a careful evaluation of their inhibition efficacy using sensitive measurement methods indicated little or no AQP1 inhibition by tetraethylammonium or acetazolamide, and apparent inhibition by DMSO resulting from an osmotic clamp artefact rather than bona fide inhibition (Yang et al., 2006a). A recent paper reported strong AQP4 inhibition by multiple antiepileptics (Huber et al., 2007), though we were unable to verify their findings using appropriate assays (unpublished results).

The general approach for identification of new drugs, such as AQP inhibitors, is high-throughput screening in which large collections of diverse synthetic or natural small molecules is screened for their activity against a target (reviewed in Verkman, 2004). Active compounds are characterized for their potency, specificity and pharmacological properties, and optimized by testing chemical analogues. In addition to usual requirements such as good pharmacology, toxicity and stability properties, AQP4 inhibitors must penetrate into brain. Potential assays to identify AQP inhibitors have been developed, as discussed elsewhere (Verkman, 2001), and include an erythrocyte lysis assay that has been successful to identify nanomolar-potency inhibitors of a urea transporter (Levin et al., 2007). It remains to be established which assay(s) of AQP function are sufficiently

robust for high-throughput drug screening, and whether AQP4 is a 'druggable' target suitable for discovery of high potency small-molecule inhibitors. Further, because AQP4 water transport function is probably already maximal, a search for small-molecule activators is unlikely to be successful; rather, transcriptional-level upregulators are likely needed to increased AQP4 water permeability.

Perspective

Phenotype analysis of AQP4 knockout mice has provided compelling rationale for the development of small-molecule AQP4 modulators as new therapeutic options for brain swelling, glial scarring and epilepsy. Though the identification and validation of a new drug target for brain disorders is potentially very exciting, particularly where therapeutic options are limited and often inadequate as is the case for brain swelling, there are significant challenges in implementing the idea of pharmacological AQP4 modulation. Discovery of potent and selective small-molecule AQP4 inhibitors is a major challenge, particularly with the requirement of efficient brain penetration. Of far greater challenge is the discovery of selective, small-molecule enhancers of AQP4 expression. AQP4 inhibitor therapy for brain swelling requires care because of complexities in cytotoxic versus vasogenic oedema. For example, AQP4 inhibition may be beneficial early in the course of stroke and head trauma, but of little utility or even deleterious later on. Similarly, AQP4 inhibition represents a two-edge sword in epilepsy therapy, as it increases seizure susceptibility as well as seizure severity. Notwithstanding these caveats, we consider the potential of AQP4 modulator therapy to be high and thus efforts focused on its implementation well justified.

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Subject Index

ACTH, 49, 142, 207, 253, 259, 274, 276, 280, 286–293, 299, 303, 305, 312, 329, 331–332, 335–342, 344, 346, 349, 355, 406–407, 411, 413, 417, 471–478, 548, 550–551, 556, 560, 564
ACTH secretion, 49, 274, 286–288, 290–291, 293, 312, 335–336, 476, 560
Actin, 70–72, 495–499, 515, 517, 519, 528, 536
Adenosine, 38, 127, 130, 132–134, 171–172, 473, 478, 508, 511, 517, 587, 592
Adrenalectomy, 278–280, 286, 289, 291–293, 318, 559
Adrenergic, 76–77, 82, 106–107, 217, 254, 278, 331, 581, 587, 589
Affinity state, 101, 106–107
Afterdepolarization, 127–128, 134
Afterhyperpolarization, 127, 134
Aggression, 3, 6, 8–9, 11, 143, 181–183, 185–189, 191, 208, 212, 214, 225–226, 228–232, 234, 238, 253, 255, 257–258, 261, 396, 398, 406, 426–427, 432, 548, 559–561, 564, 568, 570, 573, 576–577
Agonist, 21, 23, 32, 49, 67, 76, 78, 86, 89–90, 101–103, 105, 107, 131, 150, 165, 184, 200–201, 203–206, 208–209, 213, 265, 299, 314, 335, 339, 347, 351–352, 355, 405–406, 408, 411–413, 416, 418–419, 421, 427–428, 433, 445, 447–448, 461, 471–474, 511, 547–549, 551, 589, 593, 595
Allopregnanolone, 85, 88, 91, 93, 97, 297–298, 303–305
Alzet osmotic mini-pumps, 461, 463, 466–467
Amino acid replacement, 241
Amygdala, 52, 115–122, 144, 181–185, 188–189, 191, 211, 213–219, 228, 231, 234, 237–238, 254–255, 258–261, 263–264, 321, 325–326, 331, 334, 339, 350–352, 368, 384, 386, 395–400, 562, 564, 568, 575
Antagonist, 7, 10, 39, 48–50, 69–70, 76, 78, 82, 86, 88, 93, 102–104, 107, 119, 129, 131, 143–144, 155, 165–167, 170, 172, 184, 186–187, 189–190, 199–201, 203–205, 207–209, 215–216, 218, 225, 228–229, 234, 236–238, 254, 258, 286, 290–293, 298, 301, 305, 312, 314, 316–317, 323–324, 339–340, 344, 350, 354–355, 385–386, 390, 405–408, 410, 415–428, 430–433, 445, 447–448, 451–452, 455–456, 461–462, 466–467, 471, 473, 478, 486–487, 490–492, 510–511, 527, 540, 547–549, 551–556, 564, 575–576, 588, 590–591, 593
Antidiuresis, 86, 127, 158, 483, 503, 510, 525, 531
Anxiety, 9, 11, 115–117, 119–122, 128, 143–144, 181, 183, 186–191, 211–212, 214, 216–219, 252–255, 257–263, 265, 276, 326, 349, 353–355, 368, 387, 390–391, 395–398, 400, 406–407, 427, 429, 432, 473, 478, 547–548, 553, 555, 560, 568, 579
Apelin, 127, 130–133, 263, 503–511
Approach behaviour, 265, 337–338, 340–341, 344–346
AQP4, 484, 533–543
Aquaporin-2 (AQP-2), 515, 525–526
Aquaporin/s, 515, 571, 589–591, 598
Aquaretics, 418, 426, 431, 433, 483
Arginine vasopressin 1a receptor (AVPR1a), 241–242, 367–368, 559, 562
Arginine vasopressin (AVP), 3, 64, 88, 149, 181, 242, 251, 257, 321, 367–368, 383–384, 471–472, 483, 503, 515, 525, 575, 587
Astrocyte, 17, 20, 22–23, 455, 536
Astroglia, 63, 533–540
Asynchronous, 27, 29–33
ATP, 23, 94, 132–134, 152, 163, 171, 496, 517, 587–594, 596–599
Attachment, 109, 208, 218, 257, 260, 264, 390, 396, 520

Autism, 144, 187, 191, 211, 213–216, 218–219, 255, 257, 261–262, 265, 367–371, 374, 378, 383–384, 386–388, 391, 395, 398–400, 406, 411, 429, 570
Autism spectrum disorders, 187, 191, 218, 370–371, 383–384, 388, 391
Autonomic nervous system, 218, 251–252, 254, 398, 573
Avpr1a, 241–249, 367–370, 372–378, 395, 399–400, 559, 562, 564

Bed nucleus of the stria terminalis, 3, 6, 12, 17, 52, 117, 122, 138, 144, 181, 191, 207, 209, 229, 231, 254, 261, 271, 309, 311, 321, 334, 389, 402, 562
Behavior, 208–211, 266, 367
Bivalent, 405–406, 408, 413, 423–424, 432–433
BOLD imaging, 225, 234
Brain slices, 63, 67, 72
Brain stem, 116, 120–121, 182, 252, 384, 395–396
Bungarotoxin, 101, 108–109
Burst, 27–28, 32, 63, 65–69, 71–72, 128, 130–133, 374, 509

Camk2a, 567, 569–570
Cannabinoids, 37, 40, 313, 316–317, 329–330, 335–336, 339–340, 354–355, 547, 551
Carotid ligation, 451–456
CB1, 38–41, 107, 313–314, 316–317, 329–330, 332–356, 551, 554–556
Cell migration, 140, 533–540
Central amygdala, 115–117, 120–122, 181–184, 188–189, 191, 254, 398
Cerebral oedema, 451–452, 454, 456, 533
Cholesterol, 86–87, 101, 104, 106–107
Conditional, 332–333, 345, 567–569
Contraction in vitro assay, 75
Cortex, 28, 52, 88, 94, 152, 163, 198, 216–217, 231, 234, 237–238, 252, 258, 311, 325, 331–332, 334–336, 339, 351–352, 354, 385, 390, 451, 453, 509, 540–541, 560, 564
Corticosterone, 117, 227, 253, 276, 279, 290–291, 303, 311, 313, 329, 331–332, 335–336, 339–342, 344–355, 472, 474–478, 547–552, 555–556, 560
Corticotrophin releasing hormone, 29, 133, 311, 318
Corticotropin releasing hormone, 353
Cre, 333, 529–531, 567–570, 582

D2-like dopamine receptors, 197
Delivery, 7, 41–42, 95, 101, 163–166, 169, 171, 173, 188, 218, 433, 467, 533, 574
Dendrite, 56, 127, 129
Dendritic release, 11–12, 31, 40, 45, 66, 94, 120, 127, 131, 208, 275–276, 508, 510
Depression, 19–21, 23, 50, 144, 171, 215, 253, 281, 329–330, 351–355, 368, 372, 387, 427, 432, 471–473, 478, 540–541, 560
Development, 52, 75, 80, 82, 85–88, 90, 92–96, 118, 138–140, 143–144, 163–164, 168–169, 191, 229, 236, 252, 262–265, 286, 290, 330, 335, 339, 344, 351, 353, 355, 391, 406, 408, 410–411, 415, 418–419, 421, 424–433, 445, 451–452, 454, 471–472, 483, 495, 542–543, 567–569, 578–579, 592, 599
Diffusion, 17–21, 23, 57, 142, 166, 169, 199, 252, 322, 330, 422, 448, 484, 540–541
Diuresis, 41, 158, 456, 483, 503, 505–506, 510–511
Dorsal root ganglia, 64, 85, 93
d-serine, 17, 21–23, 32
Dynorphin, 127, 130–131, 302

Electrical activity, 17, 38, 40, 45, 49–50, 52, 55, 57, 70, 130, 149–150, 158–159, 198, 301, 503, 506, 509–511
Electrophysiology, 40, 63, 226, 345, 583, 593
Elongation factor, 75, 82
Endocannabinoids, 37–38, 42, 96, 311–314, 316–317, 329–333, 335, 339–340, 345–347, 349–350, 352–353, 355
Endogenous opioids, 297–298, 301–305
Epilepsy, 533, 543, 564
ERK5, 75, 77, 80–81
Estradiol, 52, 87, 97, 138–139, 211, 214, 219
Estrogen, 52, 253
Evolution, 1, 3–8, 11–12, 46, 48, 142, 144, 182, 225, 241, 243, 248, 370, 372, 378
Experimental therapeutics, 383

Fear, 51, 115–117, 119–122, 211, 216–218, 228, 252, 254, 258–260, 324, 331, 350, 352, 375, 390, 395–396, 398, 400, 579
Fetus, 83, 577
Firing pattern, 27, 31, 53, 63, 65, 67, 149–151
fMRI, 119, 217, 226, 228, 231, 234, 238, 260, 265, 386, 390–391, 395, 397–398
Foetus, 243

G-protein coupled receptors, 54, 155, 204
GABA, 18–21, 23, 29, 40, 48, 55, 85–86, 88–89, 93–95, 97, 101, 117–118, 132, 134, 163–166, 168–170, 173, 205–206, 208–209, 219, 273, 277–280, 303, 305, 311, 313, 315–318, 329, 345, 347, 461–462, 466–468, 510, 540, 589
$GABA_A$ receptor, 69, 85–90, 94–95, 164, 206, 303–304
Galanin, 38, 107, 121, 127, 130–131, 133, 140
Gene expression, 8, 54, 144, 150, 171, 205, 218, 242, 273, 275–277, 369, 461–462, 464, 467, 525, 528–529, 559, 567, 569, 594–598
Glucocorticoid, 41, 49, 169, 273, 277, 279–280, 292–293, 311–318, 332, 334, 345–347, 351, 472, 478, 483, 486–487, 491, 525, 527
Glutamate, 17–21, 23, 27–33, 38, 41, 55, 66, 69, 72, 95–96, 107, 132, 150, 155, 159, 273–274, 277–280, 311, 313–318, 329, 334, 345–347, 461–462, 467–468, 588–591, 598

Hemorrhage, 76, 93
Heteronuclear RNA, 288, 461, 465, 468
Hippocampus, 21, 23, 30–31, 39, 116, 141–142, 163–164, 166–167, 172, 182–183, 188, 203, 205, 228, 231, 233–234, 237, 311, 321–322, 325–326, 331–332, 334, 345, 351–352, 384, 400, 505, 541, 559–560, 564, 568
HPA, 253, 259, 273–275, 277–281, 286–287, 289–291, 293, 298–299, 303, 305, 311–312, 318, 329–337, 339–341, 344–355, 471–473, 475, 478, 547–548, 550–551, 556
Hyena, 138
Hyponatraemia, 451, 539
Hypothalamic explant, 132, 149, 151, 154, 159, 201, 210, 299, 313
Hypothalamic neurones, 85
Hypothalamic paraventricular nucleus, 96, 127, 181, 210, 258, 273–274, 286
Hypothalamic slice, 21, 23, 52, 86–87, 149, 152, 159, 312, 589
Hypothalamus, 3, 5, 12, 27, 30, 37, 45–54, 56–57, 64, 66–67, 87–88, 94–95, 97, 115–116, 120–121, 142, 149, 151–154, 156–158, 169, 181–182, 185, 189, 197–202, 207–209, 211, 214, 228–229, 231–235, 237, 252, 273, 303, 311–312, 315, 321–322, 331, 335–336, 339, 347, 351–352, 355, 383–384, 462, 472, 504–506, 556, 567–568, 573–574, 581, 592

Imaging genetics, 395, 400
Immunocytochemistry, 8, 70, 141, 149–151, 185, 505
Ingestion, 47, 547, 549, 553, 556
Interleukin-1β, 297, 299, 305
Intracellular calcium, 40, 50, 76, 85, 105, 116, 164, 205, 508–510
Intracellular signalling cascade, 63, 70, 594
Isolation, 48, 251, 253–255, 352, 579–580
Isotocin, 3–4, 12, 46–47, 142

Knockout, 41, 144, 169, 185, 216, 258, 290–292, 332–333, 335, 341–345, 347–349, 351, 373, 386, 399, 533, 538, 543, 547–548, 550, 552, 554–556, 559–560, 564, 567–568, 570, 573, 577

Lactation, 17–18, 21, 23, 37–38, 41–42, 46, 51, 65, 67, 86, 102, 172, 188, 191, 218, 236, 252, 259, 278, 297–298, 318, 461, 506, 567–568, 587
Learning, 116, 138, 142, 212–214, 216–218, 237, 242, 321–326, 350, 352, 384, 396, 575
Localization of oxytocin receptor expressing neurons, 573
LTP, 17, 22–23

Magnocellular, 5, 17–19, 21, 27–28, 30, 32, 37–38, 40–42, 45–47, 49–51, 53–54, 56–57, 64–66, 72, 95–96, 101, 115, 132, 142, 149, 160, 184, 198, 201–203, 206, 208–209, 273–281, 287, 289, 297–302, 304–305, 311–318, 332, 345, 347, 351, 384, 462, 468, 503–508, 510–511, 567–568, 574, 590, 593
Magnocellular neurons, 17, 19, 21, 32, 37–38, 40–42, 45–47, 49–50, 53–54, 56–57, 96, 101, 132, 142, 273, 275–279, 297, 299, 311–318, 347, 351, 384, 503–504, 506–507, 510–511, 567–568, 574, 590, 593
Male aggression, 181–183, 185–187, 191, 573
MAP kinases, 75–76, 80, 85
Maternal aggression, 181–183, 188–189, 191, 208
Maternal behaviors, 17, 73–76, 79–81, 85, 155–156, 168, 268–269, 278, 280, 305–306, 315–318, 332, 340, 453–454
Mating system, 3, 7–8, 12, 241, 244, 247–248
Medial amygdaloid nucleus, 138
Medial preoptic nucleus, 142, 197, 208
Membrane trafficking, 490, 495

Memory, 116, 119, 138, 142, 144, 187–188, 242, 257, 260, 321–326, 349–350, 352, 373–374, 383–384, 386, 390, 559–562, 564, 568, 570, 574
mEPSC, 311, 314, 318
Mice, 7, 42, 50–52, 87, 94, 102, 139–140, 144, 149–151, 153–154, 156–159, 169, 182, 185–187, 199, 209, 211–216, 218, 229, 242, 247, 254, 258, 290–292, 324, 332–333, 335–338, 340–353, 356, 373–374, 385–386, 390, 396, 399, 447, 498, 504, 510, 533–535, 537–538, 540–541, 543, 547–556, 559–564, 567–570, 573–583
Microdialysis, 66, 142, 181–184, 186–191, 235, 322–324, 332–333
Microtubule, 515, 519–520
Microtus, 7, 241–245, 247–248, 251
mIPSC, 311, 315–316, 318
Molecular genetics, 368, 435–437, 445, 446
Mouse, 41, 47, 91–93, 97, 149–151, 153–159, 212–213, 215, 243, 245, 248, 276, 290–291, 333, 345, 348, 352, 373, 386, 396, 472–473, 497, 504, 508, 518, 530, 533, 537–541, 549–551, 553, 556, 560, 562–564, 567–568, 570, 575–577, 582–583
Music, 367–368, 372–374

Neonate, 163
Neurohypophysis, 5, 17, 38, 41, 45–47, 50, 53–57, 64, 72, 85–86, 96, 149–150, 157–158, 184, 205, 274, 462, 504, 506, 509–510
Neuropeptides, 3, 9, 12, 38, 48, 51, 69, 85–86, 91–92, 115, 117–122, 127–129, 132, 138, 181–182, 184, 188, 191–192, 197, 199, 207, 212, 215, 218, 225–226, 251–252, 255, 257–258, 263–265, 275, 322, 331, 367–368, 370, 373–374, 378, 384, 390, 395–400, 462, 503, 509–510, 547, 587–588
Neuropeptides colocalization, 503
Nitric oxide, 38, 41, 49, 51, 127, 133, 204–205, 207–210, 316, 318, 345, 347, 517
Non-genomic effect, 177–178, 418
Non-synonymous substitution, 241
Nonpeptide, 420, 422, 424, 426, 428, 430, 432, 488, 490, 492, 494, 496, 498, 500, 511
Noradrenaline, 33, 38, 49, 51, 297, 299–303, 305, 331
Norepinephrine, 216–217, 219, 278, 312, 318, 509, 511, 548, 587–588
Nucleus tractus solitarii, 49, 297, 305
Org, 369, 425, 427, 432, 448
Osmosensitivity, 149, 154, 158–159
Osmotic regulation, 150, 159, 461, 483
OT, 27–28, 53, 93–94, 101–104, 106–107, 109, 167, 170, 185, 211–212, 214–216, 218–219, 399, 405–413, 415–417, 419–424, 427–433, 445–448, 473, 478
Oxytocin, 1, 3–4, 12, 17, 23, 27, 37, 40, 42, 45–57, 63, 72, 75, 77, 83, 85, 88, 91–94, 97, 101–109, 115–122, 127–128, 133, 138, 144, 149, 160, 163, 166–173, 181, 191–192, 197–198, 201–202, 204–205, 207–212, 214, 225, 234, 237–238, 251, 253, 257–258, 260, 271, 273, 297–305, 311–312, 317–318, 322, 332, 345, 347, 365, 367–368, 374, 383–386, 389, 391, 395–400, 403, 405–406, 409, 419, 421–424, 427, 429–430, 445, 461–462, 466, 468, 473, 478, 505, 507, 515, 517, 547–548, 550, 552, 554–556, 567, 570, 573–577, 580, 583, 585, 587
Oxytocin receptors, 45–46, 51–53, 63, 73, 101, 103, 106, 115–118, 167, 172, 175, 300, 347, 395–396, 421–422, 435, 445

Paraventricular, 3, 5, 12, 17, 23, 27, 37, 42, 49, 54, 64, 72, 96, 101, 115, 120, 127, 131–133, 141, 149, 160, 181, 183, 192, 197–198, 204, 207–211, 214, 234, 253, 258–259, 273–274, 276, 286–287, 297, 305–306, 311, 319, 331, 339, 383–384, 391, 464, 472, 478, 504, 512, 551, 556, 560, 567–568, 570, 574
Paraventricular nucleus, 3, 5, 12, 23, 37, 42, 49, 54, 96, 127, 131, 133, 141, 160, 181, 183, 192, 197–198, 207–210, 214, 253, 258–259, 273–274, 286, 297, 306, 311, 319, 331, 339, 384, 391, 464, 472, 478, 512, 556, 560, 570, 574
Parvocellular, 5–6, 29, 46, 49–51, 53–54, 57, 96, 115, 197, 201, 203–206, 208, 273–279, 286–291, 293, 311–312, 315–316, 331, 334–335, 339, 345–346, 351, 472
Phosphorylation, 75, 78–81, 106, 170, 173, 205, 207, 275, 484, 497–498, 511, 515–520, 595, 597–598

Pituitary mitogenesis, 286, 292
PKA, 312, 315, 317, 319, 497, 511–512, 515, 517–519
Plasticity, 3, 6, 21–23, 27, 31, 86, 116, 159, 273, 278, 280–281, 318, 355
Plateau potential, 31–32, 67, 127–128, 131, 157–158
Polymorphism, 119, 241–243, 245–246, 248, 372, 374, 387
Prairie vole, 7, 47, 138, 140, 143–144, 185, 190, 251, 253–255, 260, 368–369, 372, 385–386, 399
Preautonomic, 273, 276, 278–279, 281, 318
Pregnancy, 65, 76, 81, 95, 144, 188, 209, 219, 229, 297–298, 300–305, 318, 385, 483, 492, 547, 549–551, 553, 562
Presynaptic, 19–20, 23, 27–32, 37–41, 69, 72, 131, 205–207, 305, 311, 313–314, 317, 331, 334, 346, 510, 587, 589–591
Progesterone, 52, 85–87, 91, 97, 209, 211, 219, 229, 297–298, 303, 305, 575
Prolactin-releasing peptide, 45, 49
Promoter activity, 525, 530–531
Pup suckling, 23, 225–226, 237–238
Purinergic, 587, 589, 591–592
PVN, 3, 5, 11–12, 17–18, 23, 27–31, 33, 37, 41–42, 49–50, 52–54, 66, 141–142, 149–150, 160, 181, 183–184, 188–192, 197–207, 214–215, 217, 231, 234, 236, 238, 253, 273–281, 286–289, 292, 297, 299–306, 311–313, 315–319, 322, 331–332, 334, 339, 345–347, 349, 355, 384, 391, 464, 472, 478, 504–508, 510, 512, 551, 556, 567–568, 570, 574, 581, 593

Radiolabelled, 66, 290, 406, 408, 421–423, 429, 431–433, 474, 476, 489–491, 497, 499–501
Retrograde messengers, 37, 96, 311, 313, 317–318
Rhythmic activity, 127
Rimonabant, 329, 332–333, 353–355, 551–552
Rodents, 8–9, 11, 115–116, 144, 168, 198, 200–201, 216, 228, 236, 241–248, 251, 254, 259, 264, 287, 301, 313, 321, 323, 331, 335, 340, 386, 472, 503, 505–506, 547, 579–580

Selectivity, 105, 234, 410–411, 416, 420–421, 423–424, 427–428, 445–448, 471, 473, 478, 505
Septum, 3, 6, 10, 12, 119, 138–143, 181–183, 185–189, 191, 216, 229, 236–237, 254–255, 259, 321–326, 352, 385–386, 505, 560, 562, 583
Serotonin, 217, 219, 225, 372–373, 385, 471, 479, 497, 564, 598
Sex chromosomes, 7, 138, 140
Sex differences, 6, 8–9, 138–140, 142–144, 214, 219, 251, 255
Sexual behavior, 209, 261, 263–264, 268, 270–271, 277–286, 292, 294, 417–418, 420–421, 451
Sfp phosphopantetheinyl transferase, 101, 108
Shock, 288, 324, 337, 350, 497, 518, 587–588, 598–599
Social behavior, 3–6, 8, 11, 17, 22–23, 168, 194, 220, 261–270, 277, 291–295, 321–323, 326, 328
Social cognition, 181, 186, 191, 257, 260–262, 264, 383, 386–388, 390–391, 395–396, 398–399
Social discrimination, 215, 242, 573, 579
Social functioning, 262, 383–384, 386, 388–391
Social memory, 187–188, 257, 326, 383–384, 386, 390, 559–562, 564, 568
Social motivation, 375, 390, 559, 562–563
Social recognition, 9, 102, 138, 142, 144, 184, 188, 211–216, 218, 257, 323–326, 386, 391, 396, 559, 562, 564, 567, 570, 575, 579
Social recognition memory, 138, 142, 144, 323, 325–326
SON, 5, 17–23, 27, 30–32, 37–42, 49–51, 53–54, 56, 64–70, 72, 85–89, 91–97, 141–142, 149–160, 181, 183, 188, 192, 198–204, 206–207, 234, 236, 253, 274–275, 277–278, 280–281, 287, 297, 299–306, 311–314, 316–319, 322, 347, 384, 391, 461–464, 466–468, 504–508, 510, 512, 567–568, 570, 574, 588–595, 597–598
Species difference, 4, 7–8, 10, 243, 408, 410–413, 421, 424, 428, 430–431, 433, 445
Spillover, 17–18, 20–21, 55, 317–318
SRX251, 225, 229–234, 238, 258, 424–426, 432
Steroids, 85–87, 89, 93–96, 101, 139, 143, 229, 303–304, 321, 335
Stress, 1, 3, 11, 41, 47, 51, 80, 94, 142, 169, 186, 189–191, 213, 216, 219, 227–229, 253, 257–261, 264–265, 271, 273–281, 286–291, 293, 297, 299–303, 311, 318, 329–332, 334, 336–339, 341–345, 348–355, 383–384, 390,

406–407, 413, 427, 454, 464, 467, 471–473, 478, 547–553, 555–556, 559–560, 568
Supraoptic nucleus, 5, 17, 23, 37, 42, 45, 49, 54, 63–64, 72, 85–86, 97, 120, 127–133, 141, 149, 160, 183, 192, 197–198, 207, 209, 234, 253, 274, 297, 306, 312, 319, 332, 347, 383–384, 391, 461, 464, 468, 512, 570, 574, 588

Temperature, 51, 151–152, 452, 463, 465, 547–553, 555–556, 573, 580–581
Testosterone, 138–140, 143, 212, 214, 219
Tetrahydrocannabinol, 329
Thermo regulation, 573
Tonicity, 525, 529, 531
Transgenic, 47, 216, 242, 333, 533, 567–570, 581
Transgenic mouse, 47, 533
Treatment, 49, 52, 76, 78, 90, 102, 139–140, 186, 189, 230, 232–233, 263–265, 302–304, 323–324, 338–339, 341–342, 344–345, 348, 350, 352–354, 375, 383, 386–388, 390–391, 398–399, 405–407, 411, 425–433, 451, 455–456, 461, 466, 472–478, 490, 519, 537, 542, 547–548, 553–554, 560, 596
Trophic action, 75

Urinary concentrating mechanism, 515–516, 518

Vagus, 120–122, 251
Vaptans, 405–406, 426, 432
Vasopressin, 1, 3, 12, 17, 23, 27, 37, 42, 46–47, 49–50, 53, 55–56, 64–65, 72, 75–76, 78, 85, 88, 91, 97, 102–104, 115–122, 127–134, 138, 144, 149, 160, 181, 191–192, 199, 208–210, 212, 225, 228, 232, 235, 241–242, 249, 251, 253, 257, 261, 271, 273–274, 281, 286–287, 299, 311–313, 317–318, 321, 326, 331, 345, 347, 365, 367–368, 373–374, 383–384, 386, 391, 395–396, 398–399, 403, 405–406, 409–414, 416–417, 421–422, 424–425, 428, 430, 445, 451, 456, 461–462, 464, 466, 468, 471–474, 478–479, 481, 483–488, 490–492, 497–498, 503–505, 507–511, 515–518, 525, 559, 562, 564, 567–568, 575–577, 583, 585, 587
Vasopressin receptor antagonist, 225, 451, 483, 492
Vasopression 1b receptor, 37–38, 40–41, 106–107, 115, 119, 134, 182, 192, 252, 265, 277, 287–289, 372, 406, 408, 411–414, 417, 421–423, 427–428, 430–432, 471, 473–475, 478, 559–560, 564
Vasotocin, 3–4, 6, 8, 12, 46–47, 93, 97, 102–103, 138, 142, 144, 182, 191, 207, 374, 407, 410, 419
Ventromedial hypothalamus, 45, 53–54, 182, 237
VP, 141, 212, 214–218, 286–293, 410, 412–413, 415, 417, 420, 422, 424–427, 447, 587, 592
V_{1B} receptor, 115, 119, 134, 182, 192, 252, 265, 287–289, 406, 408, 411–414, 417, 421–423, 427–428, 430–432, 471, 473–475, 478
V_2 receptor antagonists, 432, 455, 483–484

Water balance, 158, 503, 505–508, 510–511, 515–516, 527, 533, 535–536, 587
Water channels, 483–484, 487, 511, 515, 533
Water transport, 484, 516, 533, 540, 543