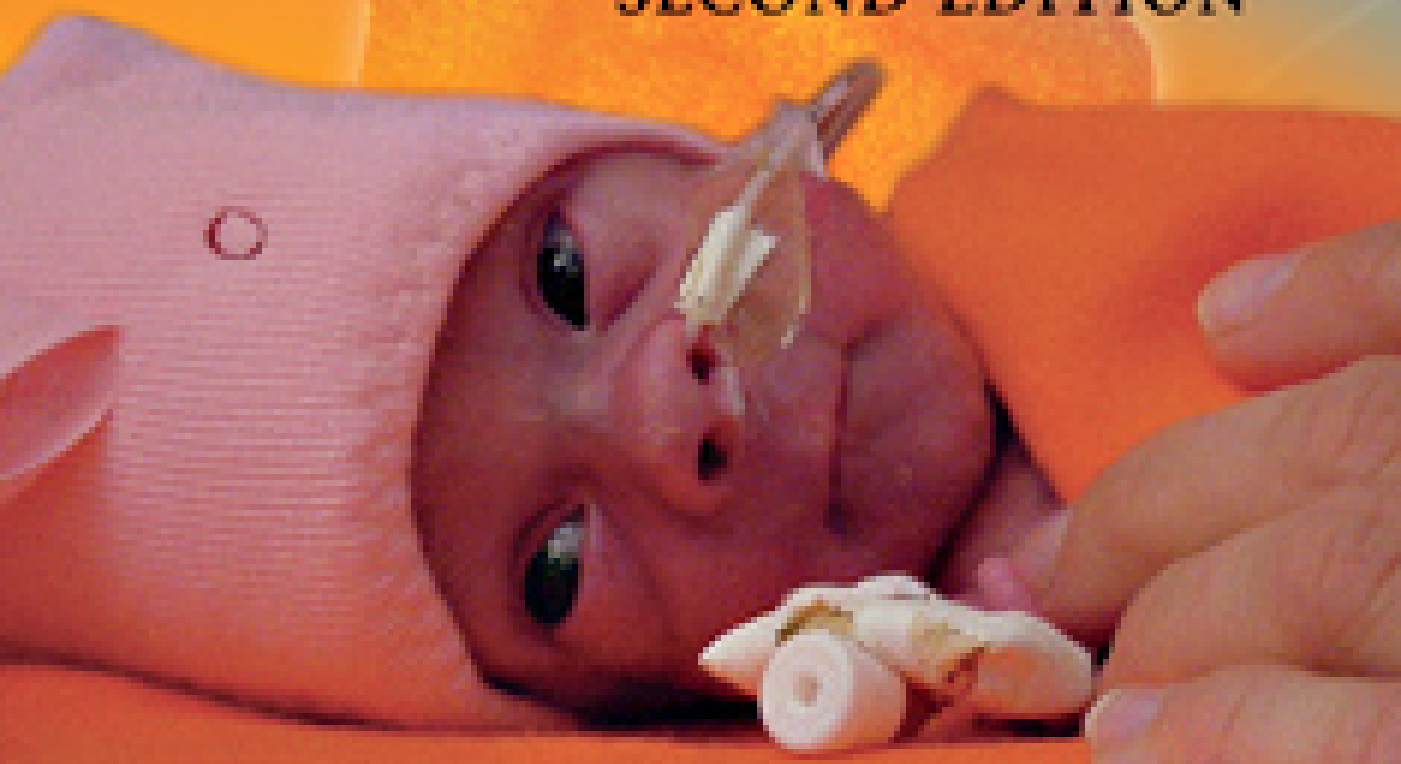
Edited by Hugo Lagercrantz, M. A. Hanson
Laura R. Ment and Donald M. Peebles

The Newborn Brain

Neuroscience and Clinical Applications

SECOND EDITION



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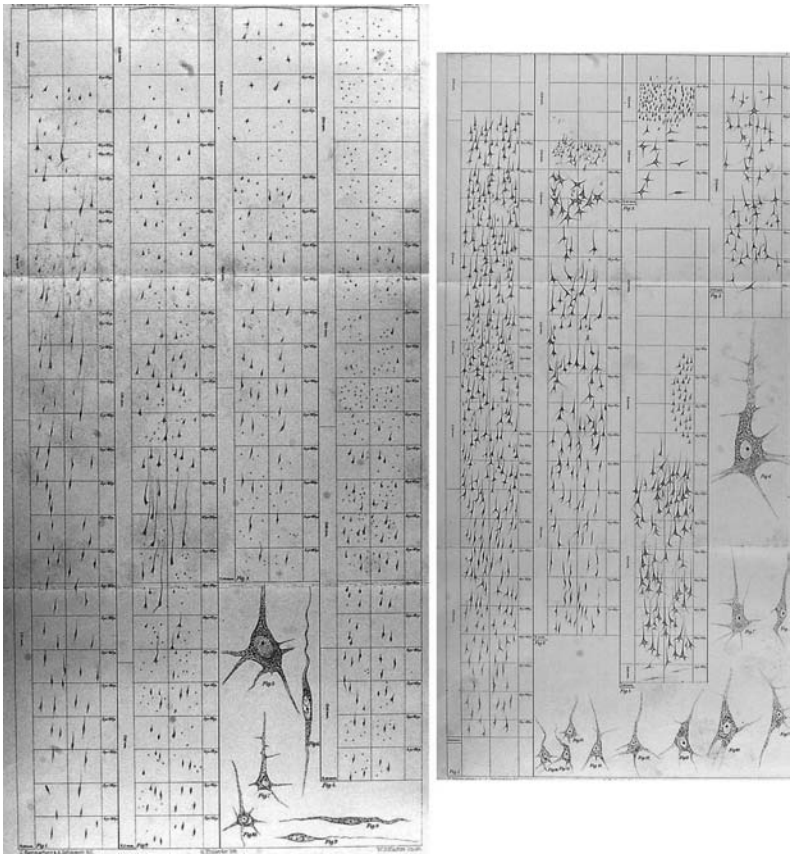
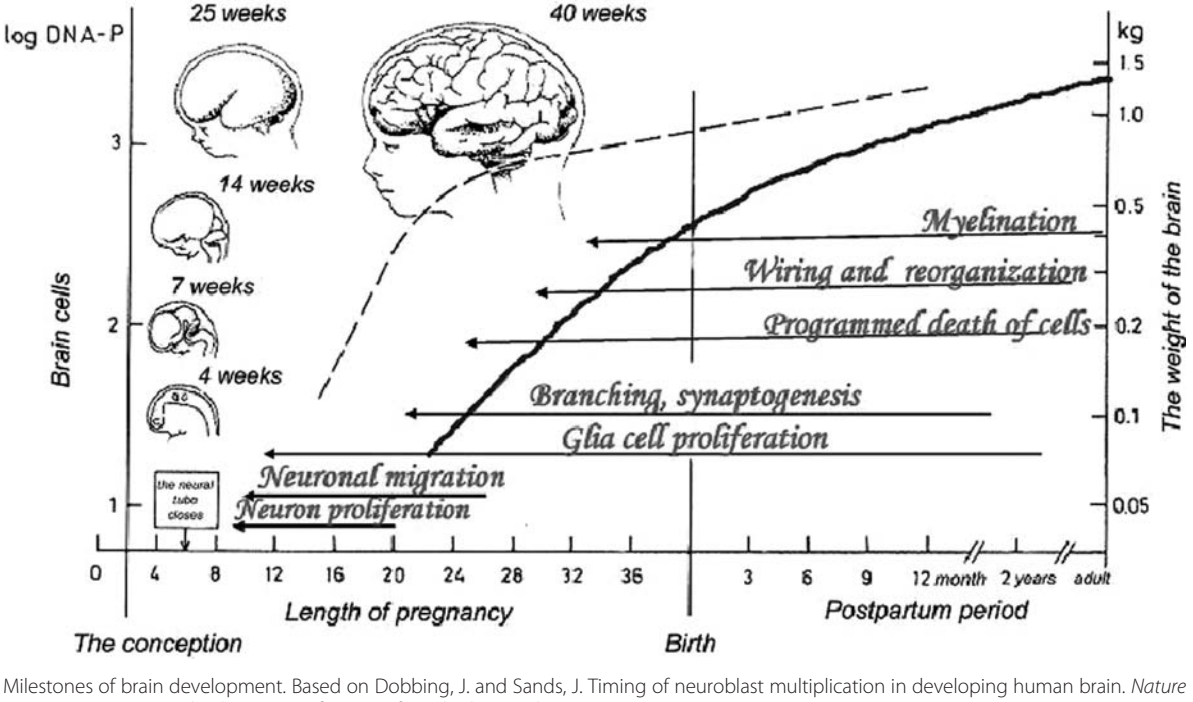
The Newborn Brain



Laminar patterns in microcephaly vera. Architectonic patterns of normal cortex (left panel) and cortex from microcephalic subjects (right panel). The Betz cells in column 3 and 4 of the left and right panels identify the pre-Rolandic gyrus in normal and microcephalic brains. The other columns in each panel illustrate corresponding regions of frontal, parietal, and occipital association cortical regions. The microcephalic cortex is laminated with attenuation of superficial layers. (From Hammarberg, 1895; Caviness *et al.*, 2008, with permission from S. Karger AG, Basel.)



Milestones of brain development. Based on Dobbing, J. and Sands, J. Timing of neuroblast multiplication in developing human brain. *Nature* 1970; **226**: 639–40 and Rakic, P. Specification of cerebral cortical areas. *Science* 1988; **241**: 170–6.

The Newborn Brain
Neuroscience and Clinical Applications

Second Edition

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Preface to the First Edition

For ages philosophers have discussed how the brain and the mind are created. Descartes and Kant thought that true ideas are innate, while Locke and Hume claimed that the brain is a blank slate at birth. William Harvey opposed the idea that the organs, e.g., the brain, are preformed and maintained that the organs develop successively – epigenesis. Sigmund Freud who can be regarded as determinist wrote that our ideas and psychology are based on small substructures (genes). The mapping of the human genome has reinitiated a debate on the concept of preformation – today genetic determinism vs. environmental instructionism. A third alternative is the idea of selectionism or neuronal darwinism. The premature brain is a jungle according to Gerald Edelman with redundant neurons and pathways and due to environmental influences only the most suitable neuronal circuits survive (see chapter by Changeux). "Cells that fire together wire together – those that don't won't." (see chapter by Penn & Shatz).

The busy obstetrician scanning the fetal brain by ultrasound or the neonatologist monitoring the newborn brain may have limited time to ponder these eternal questions. The main reason for publishing this book is to present the state of the art on how the brain is formed. The recent breakthroughs in our understanding of the development of the brain originate from studies of invertebrates like fruit-flies or nematodes, mice or ferrets. It is difficult for the hard-working clinician attending the delivery, neonatal or neuropediatric ward to grasp this literature. On the other hand, the basic scientist may have only a vague idea of the clinical expression of mutations or disorders of neuronal migration and synaptogenesis, preterm birth or perinatal asphyxia.

Jean-Pierre Changeux commences the book with some reflections on the origin of the human brain. The chapters then follow the major milestones of brain development: formation of the neural tube, neurogenesis, migration of neurons, synaptogenesis and organization of the brain wiring. Special chapters are devoted to neurotrophic factors, neurotransmitters, glial cell biology and cerebral circulation. Then the development of sensory functions is described.

The second part of the book deals with more clinical aspects, particularly methods to investigate the infant brain by imaging and electrophysiological techniques. Two chapters deal with clinical aspects of the brain of the full-term infant and one with the preterm infant. The authors have specially emphasized how the knowledge from basic science can be applied in clinical practice.

We hope that this book is of interest for a broad readership from the more theoretical biologist, molecular geneticist and the biophysicist to the clinical fellow in obstetrics, neonatology or neuropediatrics as well as the neuropsychologist. The book can also be recommended as a textbook for graduate courses.

Stockholm, Paris, and London
December 30, 2000
The Editors

Preface to the Second Edition

Understanding the development of the human brain and the emergence of consciousness is a fundamental quest, of similar magnitude to the study of the origin of life from inorganic matter. We can talk about a "big bang" of brain development, when hundreds of thousands of new neurons are formed every minute in the fetal brain and up to one million synapses are generated every second in the child's brain. Jean-Pierre Changeux opens this book with a reflection on the origin of the human brain. The main milestones – such as the proliferation and migration of neurons, synaptogenesis, formation of glial cells – are presented in separate chapters by leading authorities in the field. The selection of neuronal pathways and the organization of the neuronal circuits are discussed by Carla Shatz and others. The development of somatosensory, visionary, and auditory modalities are described in detail. There are also separate chapters on neurotrophic agents and neurotransmitters/neuromodulators.

The remainder of the book deals more with clinical matters, while also presenting the basic science necessary to understand these problems. These chapters encompass imaging the brain, biophysical assessment of the brain, hypoxic–ischemic encephalopathy, the vulnerable preterm brain, and infections of the brain. The mechanisms leading to abnormal neuropsychological outcome are also discussed in detail.

In this second edition most of the chapters from the first edition have been completely rewritten in line with modern science and clinical practice. Some new chapters have been added, for example on behavior and the emergence of consciousness.

We hope that this book will be of interest for a broad readership from theoretical biologists, molecular geneticists, and biophysicists to clinicians in obstetrics, neonatology, and neuropediatrics, as well as neuropsychologists. The book can also be recommended as a textbook for graduate courses.

Stockholm, New Haven, Southampton, and London
October 13, 2009
The Editors

Chapter 1

Reflections on the origins of the human brain

Jean-Pierre Changeux

Introduction

Human beings belong to the biological species *Homo sapiens*. The definition of the species includes the description of the characteristic anatomy and physiology of the body, as well as of the functional organization of the brain together with the multiple facets of behaviors unique to human beings. The human brain is obviously a fascinating object of scientific investigation.

The aim of this chapter is to debate the origins of the human brain. This raises an overwhelming challenge. First of all, one should attempt to delineate what makes the human brain "human," even in the newborn, and to identify the features that distinguish it from the current living primates and from its fossil antecedents. It is intriguing, on the one hand, to find ways of specifying the universal traits of "human nature" in objective terms. On the other hand, the broad diversity between individuals, in particular as a consequence of their past and recent personal and/or cultural history, raises a second challenge. Does such diversity break the unity of the human brain within the human species?

A tension thus exists in neuroscience, as well as in the humanities, between two main lines of research: one that aims at defining the *universal* characteristics of the human species, for instance at the level of the infant brain, and the other that stresses the *variability* of cognitive abilities in adults, such as the language they speak and the social conventions they adopt. It is a formidable task to deal with these contrasting approaches with the aim of achieving a meaningful scientific understanding of the human brain, its learning capacities, and its higher functions, and it seems plausible that a realistic picture of the human brain will require the synthesis of these divergent approaches. I shall therefore consider successively these two aspects of research on the human brain.

Universality, diversity, complexity

A primary difficulty, raised by the philosopher John Searle (1995), concerns the notion of function, specially psychological function in the case of humans. One should never underestimate the conceptual and experimental predicaments posed by any attempt to singularize a behavioral or mental trait. Searle even suggests that functions are not intrinsic, but might be assigned to, or imposed on, living objects or organisms, and in particular the human brain, by the observer. This problem must be taken seriously. Even though, since Darwin, attempts have been made to eliminate teleology from the life sciences, one has to be aware of a possible observer bias in the definition of a function. The difficulty is real for the pediatrician who has to objectively evaluate newborn perceptive abilities and behavior. In my opinion, Searle's criticism could go beyond the definition of function and equally applies to the description of the anatomy or to the dynamics of the electrical and chemical activities that take place within the brain. To overcome these difficulties, a recommended strategy is to elaborate theoretical models, even within the clinical framework of pediatrics. Furthermore, the theoretical models that will primarily aim at resolving brain complexity, to my view, should not to be confined to a given discipline or technique. On the contrary, they have to systematically *bring together* well-defined molecular, anatomical, physiological, behavioral, and psychological data (see Changeux *et al.*, 1973; Changeux, 2004). In any case, the aim of the modeling enterprise will not be to give an exhaustive description of brain reality: one has to remain humble! It will, furthermore, in any case be limited by its scope and by its formulation. Moreover, one should never forget that the modeling process involves the selection of theoretical representations… by the brain of the model builder!

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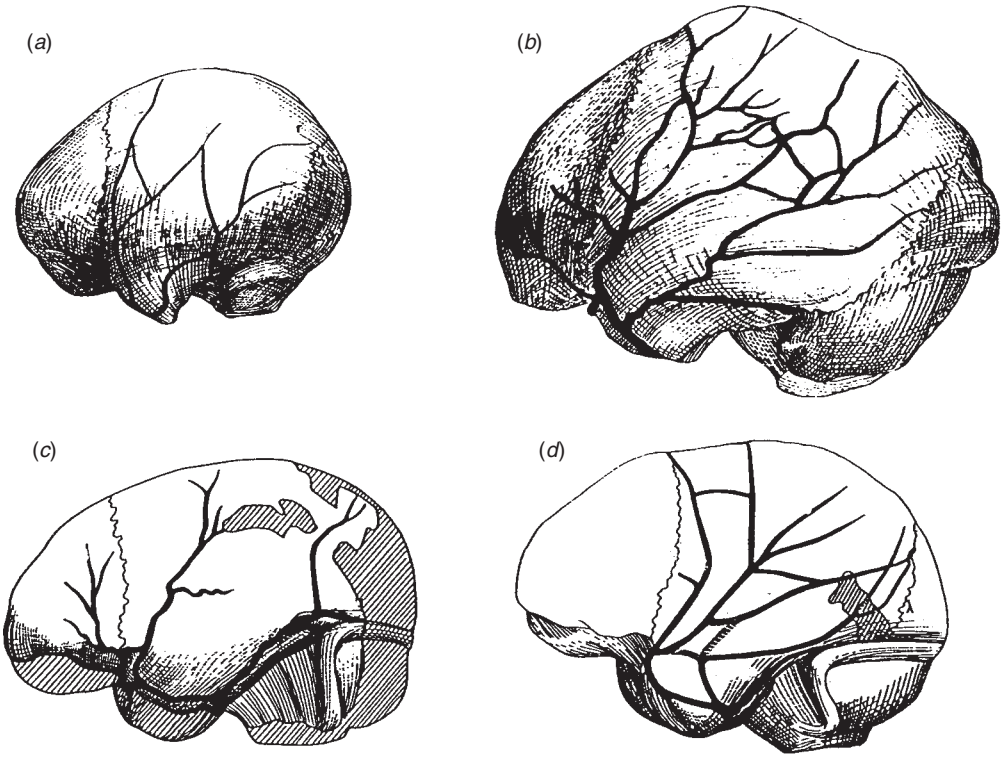

Fig. 1.1 Comparison of the impression of the meningeal vessels on endocranial casts of 40-day-old infant (*a*) and 1-year-old infant (*b*) with the endocranial casts of *Australopithecus gracilis* (*c*) and *Homo habilis* (*d*). (From Saban, 1995.)

Another difficulty resides in the very attempt to establish an appropriate causal link, or "bridge," between the structural elements of the system and the function considered. The reductionist approach, as mentioned, has to be "fair." One should deliberately avoid frequently encountered statements such as "the gene(s) of intelligence" or the "neurotransmitters of schizophrenia!" Such simplistic and incorrect proposals bypass one essential feature of brain organization; that is, there is no direct and unequivocal link between the molecular and the cognitive levels. In between these, there exist parallel and hierarchical levels of organization nested within each other and with abundant cross-connections. These levels develop step by step from the molecule to the cell, from elementary circuits to populations (or assemblies) of neurons, up to complex global neuronal patterns engaged in higher cognitive functions. The definition of these relevant parallel and hierarchical levels is in itself a difficult theoretical problem that should be made explicit (Changeux & Connes, 1989). A critical conceptual and practical issue in any investigation of the newborn brain will thus be to specify the selected hierarchical level (or, more probably, levels) of organization at which a relevant *causal link* will be established between anatomy, physiology, and behavior, together with the massive parallelism and strong lateral interactions that potentially contribute to coherent unitary brain processes.

I will make one last remark about brain complexity, which may seem far-fetched to the pediatrician: my conviction is that investigations on the human brain, in particular that of the newborn, should deal with our current understanding of biological evolution (Fig. 1.1). This requirement is obviously of practical interest, since it may lead to a fair evaluation of the commonly adopted (sometimes erroneous) use of lower animal species as models for human diseases, in particular for neuropsychiatric deficits. But the evolutionary perspective also points to interesting empirical questions. Take, for instance, the case of brain anatomy. No simple apparent logic accounts for the actual morphology, distribution, and interrelation of the multiple areas (or nuclei) that compose the brain (see Changeux, 1985). For instance, the actual nesting of the archeo-, paleo-, and neocortices within human

brain anatomy cannot be understood without considering that they have been derived by some kind of evolutionary "tinkering" (Jacob, 1981) from prior ancestral brains. The older structures have not been eliminated, but rather incorporated and nested within the newer ones. Millions of years of evolutionary history under extremely variable environmental conditions thus introduce, indirectly, *contingencies in the anatomy* such that the intrinsic logic of the functional organization of the brain may no longer be apparent by simple inspection. This situation frequently invalidates attempts to infer function from anatomy or to relate a given behavior to a single brain structure, such as, for instance, the current debates about the role of the hippocampus, amygdala, or prefrontal cortex in behavior. It also illustrates why the best models may not be the simplest or the most minimalistic ones. Model building thus has to rely on concrete observational approaches, to be "neurorealist," and it becomes a particularly difficult, though necessary, process to progress in the understanding of the human brain.

Finally, one should not limit the evolutionary perspective to the context of the biological – or genetic – origins of the human brain. Rather, as discussed in the following sections, the brain of a human subject may be more appropriately viewed as the synthesis of multiple nested evolutions by variation selection (Changeux, 1983a,b, 2004). These developments include not only the past genetic evolution of the species, but also the epigenetic development of the brain of each individual, within the framework of his or her personal history, as well as the more recent social and cultural evolution of the social environment with which the newborn interacts. The data are scarce, but the potential outcomes of future research could be richly rewarding.

Genes and the newborn brain

The brain of the newborn is often taken as holding the innate features that characterize "human nature." In reality, many more characteristics proper to the human brain develop after birth, in particular through learning during postnatal development, which is one of the longest known among living species. Even though, as we shall see, epigenetic regulations may take place which involve specific interactions with the environment, strictly innate, DNA-encoded mechanisms contribute, in a definite manner, to the prenatal and postnatal development of the adult brain (Watson *et al.*, 2007). But these genes are not expressed all at once in the egg or the embryo or the newborn, as postulated by the extreme views of the eighteenth-century preformationists, views that assumed that the adult organism was already present in a miniaturized form in the sperm and in the egg. On the contrary, they are activated (or suppressed) throughout embryogenesis and postnatal development in a sequential and combinatorial manner.

The straightforward inspection of the genetic endowment of the species compared with the organization of the brain raises, however, two apparent paradoxes (Changeux, 1983a,b, 2004; Edelman, 1987; Miklos & Rubin, 1996). The total amount of DNA present in the haploid genome comprises approximately 3.1 billion base pairs, but no more than 20 000–25 000 genes sequences (Lander *et al.*, 2001; Venter *et al.*, 2001). The coding exons represent only 1.2% of our genome, yet alternative splicing may increase the number of mRNA protein-coding sequences up to 100 000. On the other hand, the total number of cells in the brain is in the order of 170 billion, including 86 billion neurons (Herculano-Houzel *et al.*, 2007, Azevedo *et al.*, 2009), each neuron possessing its particular connectivity – or "singularity" (Changeux, 1983a). There is thus a striking parsimony of genetic information to code for brain complexity.

Another paradox is raised by the relation between the total number of genes and the evolution of brain organization. The 97 million bases that constitute the total sequence of the genome of a small invertebrate, the nematode *Caenorhabditis elegans* (Miklos & Rubin, 1996; Chervitz *et al.*, 1998; Hodgkin *et al.*, 1998; Thompson *et al.*, 2001) with its humble 302-neuron nervous system, contains a predicted 18 266 protein-coding genes. *Drosophila* possesses a much larger nervous system, with about 250 000 neurons, but with a similar number of genes (13 338; Rubin *et al.*, 2000). Even more striking, the gene number from bony fish, through the laboratory mouse, to the human is roughly constant. Yet, notwithstanding the increase of cell numbers (from about 70 million in the mouse to 86 billion in humans [Azevedo *et al.*, 2009]), mammalian brain anatomy has evolved dramatically from a poorly corticalized lissencephalic brain with about 10–20 identified cortical areas to a brain with a very high relative cortical surface, multiple gyri, and sulci and possibly as many as 100 identified cortical areas (Mountcastle, 1998). Thus, there exists a remarkable nonlinearity between the

evolution of brain anatomy and that of the total number of genes (Changeux, 1983a,b, 2004; Edelman, 1987; Miklos & Rubin, 1996).

The molecular genetics of the early stages of embryonic development in *Drosophila*, *Xenopus*, chick, and mouse offers at least one major perspective on resolving these paradoxes. For example, in *Drosophila* a variety of genes have been identified that control the cartesian coordinates of the embryo, the segmentation of the body, and the identity of its segments (Nüsslein Volhard, 1990; Lawrence, 1992). A significant fraction of these "homeotic genes," which are also found in *C. elegans* (Ruvkun & Hobert, 1998), are absent in bacteria and yeast but conserved throughout the evolution of higher animal species and possibly act in equivalent regulatory cascades in mammals. In the course of embryonic and postnatal development, these developmental genes become expressed according to well-defined spatiotemporal patterns, in a hierarchical and parallel manner with cross-regulatory interactions and reutilizations. Such a view of morphogenesis, as a developing network of gene interactions (Koentges, 2008), may account, at least in part, for the parsimony paradox. An enormous diversity, indeed, may arise from such combinatorial expression of a limited number of genes.

Development of the body plan

As a consequence of the combinatorial gene expression described in the previous section, the plan of the body's embryo develops. At defined critical stages, anteroposterior and dorsoventral polarities, and sharp boundaries between territories and/or of patterns of stripes become established. The symmetry of the embryo evolves in the course of development. "Symmetry breakings" (Turing, 1952; Meinhardt & Gierer, 1974) take place. On theoretical grounds, such defined and reproducible patterns can be generated from a set of chemical substances, or morphogens, which cross-react and diffuse throughout the organism (Turing, 1952). For instance, gradients of diffusible morphogens are thought to contribute to the unfolding of developmental gene expression resulting in anteroposterior polarity (Meinhardt & Gierer, 1974). The main factors (but not the only ones) are the products of the developmental genes: regulatory proteins referred to as transcription factors that control gene transcription at the level of the core RNA polymerase II transcription complex (see Mannervik *et al.*, 1999). These protein molecules may have played a critical role in the phylogenetic evolution of the body form (Koentges, 2008). They bind to DNA elements (enhancers or silencers) that lock or unlock the transcription of adjacent structural genes and are themselves often conserved across species. Interplay between morphogens and transcription factors (coactivators and/or corepressors) builds up an intracellular network of protein–protein interaction (Rual *et al.*, 2005; Stelzl *et al.*, 2005) and thus of gene regulation, together with membrane receptors and the relevant second messengers. Models have been proposed according to which particular sets of such molecules may contribute to the "reading" of a gradient of morphogen by some kind of all-or-none switch in both a noncellularized (Kerszberg & Changeux, 1994) and a cellularized embryo (Kerszberg, 1996) (Fig. 1.2). It has been further suggested that such reading may require particular kinds of molecular interconnections at the level of the transcription factors: the assembly of molecular partners into hetero-oligomers between, for instance, one morphogen molecule from the gradient and a transcriptional coregulator now coded by a gene expressed in the embryonic nuclei. Nonlinear relationships between transcription factor concentration and morphogenesis may thus emerge from these combinations. Such a concept of nonlinear networks of transcription factors (Kerszberg & Changeux, 1994) has been recently documented with particular reference to *Drosophila* (Mannervik *et al.*, 1999) and may plausibly contribute to morphogenesis, together with receptors, kinases, phosphatases, G-proteins, and second messengers (Lisman & Fallon, 1999) within and between the developing embryonic cells (Koentges, 2008).

Many developmental genes are expressed in the nervous system. They are part of a still largely uncharacterized population of genes concerned with brain morphogenesis: its segregation into definite patterns of areas and nuclei and even the differentiation of asymmetrical hemispheres. The concepts mentioned for embryonic development may also apply to brain morphogenesis, in particular to the very early stage referred to as neurulation (see Kerszberg & Changeux, 1998). The process of neurulation differs strikingly in invertebrates and vertebrates. In the former case, the neuroblasts delaminate from the neural ectoderm to form progressively the ventral solid ganglion chain of the adult (which may reach up to 520 million

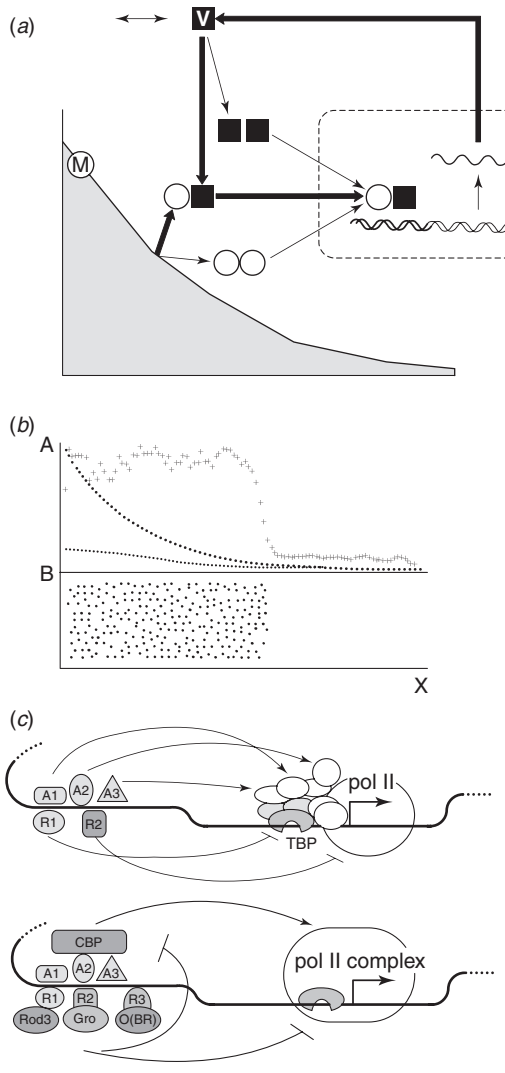

Fig. 1.2 A hypothetical model for reading morphogenetic gradients underlining the importance of protein–protein interaction and combinatorial information between transcription factors at the gene level. (*a*) A morphogen M (circles) is initially distributed along a smooth anteroposterior gradient; a 'vernier' molecule V (square) is coded by a gene present in the embryonic nucleus (broken line). Morphogen and vernier may form heterodimers but may also exist as homodimers. These various dimers form transcription factors which diffuse in the cytoplasm and bind to a promoter element regulating, in *cis*, the transcription of the vernier gene. Each dimer contributes to activation/inactivation of the vernier gene transcription thus yielding autocatalysis and competition and, as a consequence, sharp boundaries (*b*) and/or stripes (from Kerszberg & Changeux, 1994; see also Smolen et al., 2000). (*c*) Plausible schemes for the integration of combinatorial information from various transcription activators and repressors (various letters) at the level of the RNA polymerase (pol II) transcription complex (from Mannervick et al., 1999).

neurons in *Octopus*). In the latter, the neural plate invaginates en bloc dorsally to form a hollow neural tube, which may facilitate considerable growth of the central nervous system through surface expansion. This is observed from cyclostomes to mammals, primates, and humans (see also Chapter 2).

Such a decisive evolutionary step is not fully understood. Yet, on strictly theoretical grounds, one may propose the hypothesis that it does not require a large number of molecular changes at the gene transcription level. Arendt and Nübler-Jung (1997) have presented evidence that the ventral–dorsal transition of the nervous system from invertebrates to vertebrates can be reduced to simple changes in the gastrulation movements of the embryo, themselves under the control of a few homeotic genes. These discrete molecular transitions may, for instance, affect transcription factor switches, which themselves regulate cell motion (Kerzsberg & Changeux, 1998) as well as cell adhesion (see Edelman, 1987). As a consequence, either the whole neural plate infolds into a tube (in vertebrates) or individual neuroblasts delaminate, yielding a solid nervous system (in invertebrates). This illustrates hypothetically how only a few gene changes may contribute to the critical transition between the invertebrate and vertebrate nervous systems.

Another example, even less well understood, is that of the increase in size and fast expansion of the cerebral cortex and cerebellum that took place in the course of the evolution of the vertebrate brain, from fish to reptiles, birds, and mammals (see Changeux, 1983a,b; Mountcastle, 1998; Reiner *et al.*, 2004). First of all, the biochemical differences between these adult brains look negligible: from mouse to human brain, the region-specific genes are strikingly conserved at both the sequence and gene expression levels (Strand *et al.*, 2007). Moreover, in the cerebral cortex, the number of neurons per cortical column appears uniform throughout vertebrates (Rockwell *et al.*, 1980). Thus, the surface area of the cortex, i.e., the number of columns, appears as a primary target of the evolutionary changes (Rakic, 1988). One may then further speculate that the fast expansion of the frontal lobe and parietotemporal areas, which contributed to the evolutionary origins of the brain in *H. sapiens*, has resulted from the exceptionally prolonged action of some developmental genes (or of slight variation of concentration of morphogens) (Changeux, 1983a,b, 2004), the genomic

evolution underlying this process engaging a rather small set of genes.

In the course of the subsequent development of the cellular organization of the nervous system, large populations of cells project into other large ensembles of neurons, and neural maps develop in regular species-specific patterns. The molecular mechanisms governing the formation of ordered connections of neural maps are progressively being understood and, again, it is anticipated that a few molecular events will greatly modify the developmental patterns (for a review, see Tessier-Lavigne & Goodman, 1996; Drescher *et al.*, 1997). For example, in the case of the cerebral cortex, four transcription factors – COUP-TFI, Emx2, Pax6, and Sp8 – display graded expression across the embryonic cortical axes and determine the sizes and positions of cortical areas by specifying (or repressing) area identities within cortical progenitors (Bishop *et al.*, 2000; O'Leary *et al.*, 2007).

Phylogenetic ancestors of the human brain

As mentioned above, many important anatomical features of our brain have been inherited from our direct ancestors (see Changeux, 1983a, b; Mountcastle, 1998). The soft parts of their brains may be lost forever, but comparison of the endocranial casts of modern humans and their fossil ancestors provides interesting information. It reveals striking analogies between the various stages of the phylogenetic evolution of the ancestors of *H. sapiens* and the ontogenetic development of the brain in the modern human (Saban, 1995). The observations are limited to the impression of the meningeal veins and thus yield only limited information. Yet, the simplified topography of the *human newborn* meningeal system strikingly resembles the arrangement in *Australopithecus robustus* (who lived about three to two million years ago). The meningeal topography of *Homo habilis*, who lived two million years ago (cranial capacity 700 ml), is rather similar to that of a modern 40-day-old infant. *Homo erectus*, who lived one million years ago (cranial capacity of about 1000 ml), has a meningeal system topography similar to that of a modern 1-year-old child. Neanderthals (brain volume about 1500 ml, larger than modern *H. sapiens*) retained many archaic features of *H. erectus*. Recent DNA studies indeed suggest that they were only our cousins (Krings *et al.*, 1997; Carroll, 2003).

Making inferences from these rather scarce paleontological data about how spoken language and higher cognitive functions including self-consciousness emerged is a highly controversial issue. Chimpanzees use tools, have intricate social lives, and show rudiments of self-awareness (Hauser, 1999, 2005; Jensen *et al.*, 2007; Premack, 2007). They utilize a number of vocalizations, but lack rapid manipulation of symbolic representations, as well as the capacity to form and organize abstract concepts. Monkeys have been claimed to possess the equivalent of Broca's and Wernicke's areas, although without the rich connectivity that characterizes language processing in humans (Aboitiz & Garcia, 1997; Deacon, 1997; Gil-da-Costa *et al.*, 2006). An analysis of fossil skulls supports the view that the early evolution of the hominoid brain included three major reorganizations (Holloway, 1995): a relative enlargement of the inferior parietal lobe, an expansion of the frontal lobe, and a greater hemispheric specialization occurring before major increases in brain volume. Similar conclusions have been reached from a neuroanatomical perspective (Aboitiz & Garcia, 1997). This study more specifically points to the development of strong corticocortical cross-modal interactions in the postrolandic cortex, providing the basis of a semantic neural device that converges into a prospective Wernicke's area in which concepts acquire their specific link with sound. These phonological representations project into inferoparietal areas which connect to Broca's area and the premotor representations of orofacial movements. Finally, a fundamental element in the evolution of cognitive and linguistic capacity would be the coordinated operation of these networks, which is required to generate higher levels of syntax and discourse associated with the expansion of the prefrontal cortex, together with the development of its interconnections with the above-mentioned cortical (and subcortical) areas (see Chapter 23).

The morphology of the face and skull, as well as many body characteristics of human adults, resembles that of the face and skull of newborn chimpanzees (Stack & Kummer, 1962). It has thus been suggested that neoteny, i.e., access to sexual maturity at early stages of development and/or the persistence of embryonic or fetal characters in the adult, together with the prolonged development and increase in brain size after birth, contributed to the evolution of the human brain (see Gould, 1977). In any case, the

intrinsic changes in the cellular organization of the brain that make us human are already present before birth and cannot be derived exclusively from neoteny; the proliferation of nerve cells largely (but not definitively) stops at around eight months of prenatal development (except in the hippocampus and cerebellum). Without doubt, in the newborn the brain already possesses a highly organized neuronal architecture determined by an envelope of genetically coded processes.

The identification of the genetic events that have been at the origin of the brain in *H. sapiens* is still in its infancy but data that are now available on the genome and transcriptome of the rhesus monkey (Rhesus Macaque Genome Sequencing and Analysis Consortium, 2007) and the chimpanzee (Herlyn & Zischler, 2006), as well as humans (Levy *et al.*, 2007), offer interesting possibilities. From a comparative analysis it appears that about one-third of our genes started to evolve as human-specific lineages *before* the differentiation of humans, chimpanzees, and gorillas took place. This may account for the findings of very old human-specific morphological traits in the fossil record, which anticipate the recent emergence of the human species by about five to six million years (Ebersberger *et al.*, 2007). Comparative analysis further reveals that, if humans and chimpanzees show high similarity in sequence between orthologous genes, the rate of gene turnover in humans is more than two and a half times faster than in other mammals. Several gene families have expanded or contracted by shaping copy-number more rapidly than expected even after accounting for an overall rate acceleration in primates (Hahn *et al.*, 2007). Quite surprisingly, some gene families have decreased in size in the human lineage. This is the case of the olfactory genes family, of which the proportion of inactive (pseudogenes) genes is larger in humans than in other apes and larger in apes than in the mouse (Gilad *et al.*, 2000; Rouquier *et al.*, 2000). Another interesting difference is that alternative splicing is larger in humans than in chimpanzees in the corresponding tissues from the two species (Calarco *et al.*, 2007). Last, genomic surveys in humans identify a large amount of recent positive selection, to the extent that, using the 3.9-million HapMap single nucleotide polymorphism (SNP) dataset, positive selection has, unexpectedly, accelerated greatly during the past 40 000 years in human populations (Hawks *et al.*, 2007).

"Phylogenetically old" genes and neuropediatric disorders

Few of the fast-changing genes of human lineage have been investigated in detail. Particularly interesting for the pediatrician are the primary microcephaly genes that cause a reduction in brain volume to a size comparable with that of early hominids (Ponting & Jackson, 2005; Bond & Woods, 2006). Microcephalin and abnormal spindlelike-associated microcephaly are among the several genes for which mutation causes pathology by affecting neurogenic mitosis and fetal brain growth. These genes appear to alter neural progenitor cell number at a rather simple molecular target: the microtubular organization at the centrosome and the mitotic pathway. This modification may alter the action of neural progenitors when switching from symmetrical to asymmetrical cell division. The rapid evolution of these genes in the human lineage has been related to the increase in relative brain size during primate evolution (Evans *et al.*, 2005).

Childhood apraxia of speech or verbal dyspraxia is an impairment of speech production with a broad profile of linguistic deficits caused by alteration of the gene coding for the transcription factor FOXP2. In this context, an ancient DNA analysis of the *FOXP2* gene, undertaken on two Neanderthal specimens from El Sidrón, northwestern Spain, and dated to around 43 000 years ago, showed two evolutionary changes in the derived human form of FOXP2 (Krause *et al.*, 2007). Since FOXP2 has undergone recent positive selection in human history, it has been speculated that the evolution of human *FOXP2* gene might be related to the emergence of modern speech capacities (Enard *et al.*, 2002, 2009).

Other genes of relevance for the newborn brain are the genes involved in axon guidance and target selection. Most (though not all) of them have been identified in the fly and their homolog recognized in high vertebrates and humans. They comprise a finely tuned code of attractive and repulsive cues and their receptors that include, for instance, semaphorins/plexins, netrins/DCC or Unc5a-d, and slit/Robo, among many others (Tessier-Lavigne & Goodman, 1996). Interestingly, these cues also help blood vessels to navigate to their target often alongside nerve fibers (Carmeliet & Tessier-Lavigne, 2005). Other types of molecule involved in axon guidance are the cell adhesion molecules of the neural cell adhesion molecule (NCAM) and of the L1 family type that interact with

attractant and repellent guidance receptors to control growth cone and cell motility in a coordinated fashion (Maness & Schachner, 2007).

Several genes associated with autism have been recently identified. Among the many genes implicated in this disorder, particularly relevant ones are the neuroligins NLG4 and 3 and their binding partner, the scaffolding protein SHANK3, which are critically involved in synapse formation and stabilization (Persico & Bourgeron, 2006). The neurological basis for dyslexia, or reading disability, is due in large part to genetic factors. Among the several candidate genes for dyslexia (Paracchini *et al.*, 2007), *ROBO1* is orthologous to a *Drosophila* gene widely expressed in the developing nervous system. It encodes an axon guidance receptor protein that is essential for directing the outgrowth of both the axon and dendrite during development and in particular the neuronal axon crossing the midline between hemispheres. The exact functions of the other three candidates genes (*DCDC2*, *KIAA0319*, and *DYX1C1*) have yet to be elucidated, but they have all been shown to be involved in glia-guided neuronal migration during the formation of the cortex.

In short, these studies on the molecular genetics of human brain evolution have unexpected important medical consequences: more than 3000 genetic diseases that correspond to gene mutations or defects are known in humans. Many of them affect brain functions in one way or another and are already expressed in the infant brain (see Mandel, 1995). At variance with a commonly accepted "empiricist" point of view, *the brain of the newborn is not a* tabula rasa *but a richly organized structure*.

Individual variability of the human brain and the activity-dependent epigenesis of neuronal networks

The concept of synaptic epigenesis

Recent studies on human genomes carried out at the level of individuals have revealed an important inherent variability that may not systematically result in disease phenotypes. Indeed, about one base pair in 400–500 is polymorphic in the nuclear genome. Thus, two copies of the genome from different individuals will show about 1×10^6 to 2×10^6 sequence differences. The vast majority of these differences are selectively neutral but others may, as discussed, alter in a subtle way the function, or regulation, of a gene. Most of the polymorphism of the human leukocyte antigen (HLA) region was already present in the ancestors of chimpanzees and gorillas before the separation of the human lineage (Gyllensten & Erlich, 1989). Genome-scanning technologies have recently revealed an unexpectedly large extent of "structural blocks" variations in chromosomes that include deletions, duplications, and large-scale copy number variants as well as insertions, inversions, and translocations. These variants can comprise millions of nucleotides of heterogeneity within every genome and plausibly contribute to human diversity and disease susceptibility (Paabo, 2003; Feuk *et al.*, 2006). Heredity is thus often stated to be a major source of individual variability in the human brain. It may possibly account for important variations in the precise topology of defined Brodmann's areas noticed among the few brains of different individuals investigated with the required anatomical accuracy (see Mountcastle, 1998). An important variability also exists at the functional level. Joint positron emission tomography (PET) and magnetic resonance imaging (MRI) have revealed significant intersubject variability of functional areas in the visual cortex in the range of 5 mm (Hasnain *et al.*, 1998).

To analyze further the relative contribution of genetic versus "epigenetic" or environmental factors in this variability, three-dimensional maps of gray matter and models of cortical surface anatomy were derived from high-resolution three-dimensional magnetic resonance images from groups of unrelated subjects, dizygotic and monozygotic twins (Thompson *et al.*, 2001). Their comparison revealed a genetic continuum in which brain structure was found to be increasingly similar in subjects with increasing genetic affinity. Brain structures under considerable genetic control include Broca's and Wernicke's language areas, as well as frontal brain regions. In a series of anatomical (see Steinmetz *et al.*, 1995; Traino *et al.*, 1998; Eckert *et al.*, 2002) and behavioral (see Kee *et al.*, 1998) investigations carried out with genetically identical monozygotic twins who were discordant for handedness, both the in vivo measurements of the planum temporale by MRI and the results of behavioral tasks (e.g., finger tapping with anagram load) collected with left- and right-handed monozygotic co-twins yielded convergent results. The right-handers showed leftward hemispheric asymmetry, whereas the left-handers lacked symmetry. Early

epigenetic events taking place during early embryogenesis may thus contribute extensively to variability in the development of the anatomo-functional laterality of the cerebral hemispheres in genetically identical twins. In other words, "cloned" humans are not anticipated to be neurally identical.

Another rather important cause of variability originates from the way the entire network of connections becomes established between neurons during embryonic and postnatal development. The million billion (10^{15}) synapses that form the human brain network do not assemble like the parts of a computer according to a plan that defines precisely the disposition of all individual components. If this were the case, the slightest error in the instructions for carrying out this program could have catastrophic consequences. The mechanism appears, on the contrary, to rely on the progressive setting of robust interneuronal connections through trial-and-error mechanisms that, even though they are typically nongenetic, i.e., epigenetic, formally resemble an evolutionary process by variation selection (Changeux *et al.*, 1973; Changeux & Danchin, 1976; Edelman, 1978, 1987; Changeux, 2004).

Information about synaptogenesis in the developing brain may be provided by examining in adults the variance in synaptic phenotype of genetically identical individuals. The analysis has to be carried out at the level of the single nerve cell and of the exact topology of all the synaptic contacts it establishes with its partners. Such an analysis was achieved by serial sectioning of the brain of parthenogenetic animals such as the water flea *Daphnia magna* and the fish *Poecilia formosa* (see Levinthal *et al.*, 1976). At the electron microscope level, there exists a fringe of variability – a "graininess" – in the details of the axonal or dendritic branching of an identifiable neuron, the variability between left and right arborization being smaller than that found between one individual and another.

In a mammal such as the mouse – the situation might be even more extreme in humans – the number of cells is much greater and there are no longer any identifiable single cells recognizable from one individual to another. Despite common principles in gross architectural features delimited by a species-specific genetic envelope, the individual variability of the fine anatomy observed between individuals from genetically homogeneous strains increases greatly, to the extent that one wonders how such an apparently scrambled connectivity may result in reproducible behavior, even in identical twins!

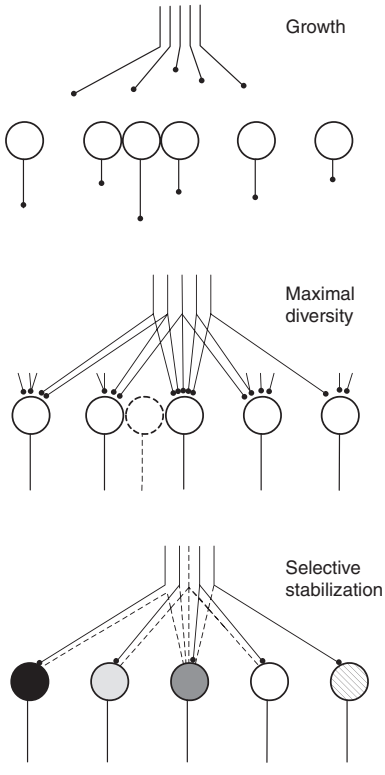

Fig. 1.3 A simple representation of the model of epigenesis by selective stabilization of synapses (from Changeux, 1983b). An interesting outcome of the formal model is that after selection different patterns of connections may yield the same behavioral input–output relationship. The different shadings of the cell bodies indicate different neuron individualities or singularities.

One plausible solution (among many others) is that the state of activity of the developing nervous system contributes to the organization of the adult network by trimming up synapse formation at sensitive periods of development. The formal model suggested (Changeux *et al.*, 1973; Changeux & Danchin, 1976; Edelman, 1978, 1987; Purves & Lichtman, 1980; Changeux, 1983a,b, 2004; Luo & O'Leary, 2005; Low & Cheng, 2006) relies on the observation that synapses do not form en masse, at once, but progressively, through exuberance followed by pruning steps, under the control of the state of activity of the developing network (Fig. 1.3). Throughout the overall development of the cortical mantle in primates and humans, several distinct phases may be recognized

(Bourgeois *et al.*, 2000; Huttenlocher & Dabholkar, 1997; see also Chapter 5).

Programmed neuronal cell death and synaptic pruning

Concomitant phases of proliferation and regression already take place in the generation of the nerve cell layers of the spinal cord, and of the cerebral cortex. The classic observation of Hamburger (1975) that about 40% of the motor neurons in the chick embryo die between the sixth and the ninth days of embryonic life is now further documented in the mouse for the cerebral cortex. Enzymes such as caspase 3 and caspase 9 must be expressed for a cell to die (see Chapter 17). Interestingly, inactivating their genes in the mouse reduces apoptosis, and increases the number of founder and precursor cells, and of radial cortical units and thus of cortical neurons. As a result, the cortical surface expands and even forms the beginnings of sulci and microgyri in this lissencephalic brain (Kuida *et al.*, 1996, 1998). The extent to which the state of activity of the developing nervous system controls such proliferative versus apoptotic steps is still being debated. Yet, correlations have been established between cell death and neuronal activation, a well-documented case being that caused by calcium entry mediated by glutamate *N*-methyl-D-aspartate (NMDA) receptors (Nicotera *et al.*, 1999). The example of the neuromuscular junction is particularly simple since only a single synaptic contact persists in the adult. On the other hand, in the newborn rat, each muscle fiber receives four or five active motor axon terminals. As the rat begins to walk, the number of these functional terminals progressively decreases until for the adult only one is left and the state of activity of the innervated muscle controls this elimination (e.g., Benoit & Changeux, 1975, 1978; Sanes & Lichtman, 1999). Axons disappear through an unusual cellular mechanism, in which they shed numerous membrane-bound remnants by engulfment of axon tips by Schwann cells (Bishop *et al.*, 2004). A similarity exists between axonal pruning and wallerian degeneration, which involves microtubule breakdown and mobilizes the ubiquitin–proteasome system (Luo & O'Leary 2005). Axonal pruning phenomena have also been documented at the synaptic level in other systems such as the sympathetic ganglia (Purves & Lichtman, 1980) or the climbing-fiber Purkinje cell synapse in the cerebellum (for reviews, see Changeux & Mikoshiba, 1978; Crépel, 1982; Kano *et al.*, 1997). For the latter, a mutation that inactivates a specific neurotransmitter receptor (the type 1 metabotropic glutamate receptor [mGluR1]) delays the regression of supernumerary climbing fiber innervation (Kano *et al.*, 1997).

The contribution of synaptic activity (evoked and/or spontaneous) to the formation of cortical circuits has been well documented since the classic experiments of Wiesel and Hubel (1963a, b), which demonstrated the important role of visual experience in fixing the organization of ocular dominance columns (for reviews, see Katz & Shatz, 1996 and Chapter 10), yet within a "functional validation" framework. At variance with this scheme, an exuberant sprouting and proliferation of axon branches, accompanied by limited although critical elimination of collaterals, has been visualized at different locations along the visual pathway (retinogeniculate, thalamocortical, and pyramidal cell arbors) at sensitive periods of development (see Stretavan & Shatz, 1986; Katz & Shatz, 1996). The state of activity of the developing cortical circuits controls synaptic evolution by more than simply validating preformed circuits. Such epigenetic regulation may concern the overall development from the early interaction with the physical world up to the elementary social experience (see Hadders-Algra *et al.*, 1996 and Chapter 21).

The model suggested (Changeux *et al.*, 1973; Changeux, 1983a, b; see also Harris *et al.*, 1997; Elliott & Shadbolt, 1998; Miller, 1998) posits that during synapse formation the genetic envelope controls, in addition to the division, migration, and differentiation of cell categories, the behavior of the growth cone, the outgrowth and formation of widespread connections, the recognition of the target cells, and the onset of spontaneous activity; it also determines the structure of the molecules that enter into the architecture of the synapse, the rules governing their assembly, and the evolution of this connecting link by the activity of the network. Yet, at sensitive periods of circuit development, the phenotypic variability of nerve cell distribution and position, as well as the exuberant spreading and the multiple figures of the transiently formed connections originating from the erratic wandering of growth cone behavior, introduce a maximal diversity that is then reduced by the selective stabilization of some of the labile contacts and the elimination (or retraction) of the others. The crucial hypothesis of the model is that the evolution of the connective state of each synaptic contact is

governed globally and, within a given time window, by the overall "message" of signals experienced by the cell on which it terminates. In other words, the activity of the postsynaptic cell regulates the stability of the synapse in a retrograde manner (Changeux *et al.*, 1973).

In this framework, the suggestion was made that reward signals received from the environment may control the developmental evolution of connectivity (Gisiger *et al.*, 2005; Gisiger & Kerszberg, 2006). In other words, reinforcement learning would modulate the epigenesis of the network. The model has been implemented in the case of the learning of a visual delayed-matching-to-sample task or delayed-pair-association task. It deals, among other testable predictions, with the developmental emergence of a "neuronal workspace" that would include the prefrontal cortex and control in a top-down manner the activity in the visual areas of the inferior temporal cortex (Gisiger *et al.*, 2005) (see the section "Representations, reward, and consciousness in the infant brain").

Trophic factors and synaptic epigenesis

Among the various consequences of the modeling approach, a simple one has been to look for the molecular mechanisms of the regulation of synaptic outgrowth, stabilization, and elimination by neural activity. At the presynaptic level, neurotrophins such as NGF, brain-derived neurotrophic factor (BDNF), NT4 and several others (for reviews, see Levi-Montalcini, 1987; Barde, 1990; Chapter 6) have become plausible candidates for retrograde signals in activity-dependent synaptic outgrowth and selection (Thoenen, 1995; Katz & Shatz, 1996). For instance, in vivo intracortical infusion of diverse neurotrophins prevents the shift of ocular dominance in favor of the eye that has not been deprived of visual experience (Maffei *et al.*, 1992; Carmignoto *et al.*, 1993; Cellerino & Maffei, 1996) on the formation of ocular dominance columns (Cabelli *et al.*, 1995). Retinal BDNF is also required in the environmental enrichment effects on the development of retinal ganglion cell dendritic stratification (Landi *et al.*, 2007). Also, rapid and opposite effects of BDNF and NGF have been demonstrated on the whisker barrel representation of the rat somatosensory cortex. Moreover, neurotrophins modulate synaptic strength within minutes in vitro at cultured neuromuscular synapses (Lohof *et al.*, 1993) and glial cell line-derived neurotrophic factor (GDNF) overexpression in the mouse delays the elimination of supernumerary motor axons and causes hyperinnervation of neuromuscular junctions (Nguyen *et al.*, 1998). Additional evidence for a contribution of neurotrophins in the epigenetic regulation of synapse development is the observation that their synthesis and release from dendrites are regulated by neuronal activity (Thoenen, 1995). Formal models of synapse-selective stabilization based on competition for limited stocks of trophic factors have been developed (Gouzé *et al.*, 1983; Harris *et al.*, 1997; Elliott & Shadbolt, 1998; Miller, 1998), giving plausibility to the theory.

On the postsynaptic side, the molecular mechanisms involved in the development of the postsynaptic domain and in the respective roles played by factors released by the afferent nerve and by electrical activity are becoming progressively understood, in particular in the simple case of the neuromuscular junction (Duclert & Changeux, 1995; Sanes & Lichtman, 1999; Changeux & Edelstein, 2005). The methods of recombinant DNA technology have led to the demonstration that, within the multinucleated adult muscle fiber, only the nuclei located under the afferent motor nerve express the subjunctional molecules such as the nicotinic acetylcholine receptor subunits. On the other hand, in the extrajunctional nuclei, the receptor genes that were transcribed in the non-innervated myotubes become repressed by electrical activity in the adult. Denervation that silences the muscle fibers causes a reactivation of transcription in the extrajunctional nuclei.

The DNA regulatory elements and transcription factors that bind to these elements and are selectively involved in the transcriptional regulation of acetylcholine receptor genes in the junctional and extrajunctional compartments have been identified, together with the intracellular second messenger systems, establishing the membrane-to-gene link (Schaeffer *et al.*, 1998). Trophic factors released from the motor nerve (such as ARIA-heregulin) bind to a tyrosine kinase receptor and initiate a cascade of signals including a MAP kinase. On the other hand, electrical activity propagated in the extrajunctional domain causes the entry of Ca^{2+} ions, and activates a serine/threonine kinase (see Schaeffer *et al.*, 1998). In both cases, a differential phosphorylation/dephosphorylation of distinct transcription factors takes place (Altiok *et al.*, 1997;

Schaeffer *et al.*, 1998; de Kerchove d'Exaerde *et al.*, 2002) together with a chromatin remodeling including histone hyperacetylation and hyperphosphorylation (Ravel-Chapuis *et al.*, 2007). Posttranscriptional regulatory mechanisms involving molecules from both the basal lamina and the cytoskeleton also contribute to the targeting, clustering, and stabilization of the receptor molecules that compose the postsynaptic domain (see Duclert & Changeux, 1995; Sanes & Lichtman, 1999; Changeux & Edelstein 2005). These posttranscriptional mechanisms become predominant in the formation and stabilization of neuronal synapses (Betz, 1998). As mentioned, the genes that make the difference between worm and yeast include, among numerous copies of tyrosine kinase, protein phosphatase, and transcription factor genes (Ruvkun & Hobert, 1998). A molecular biology of the epigenetic regulation of synapse formation has emerged.

A plausible mechanism for schizophrenia

Schizophrenia is well recognized as a brain developmental disease and it has been recently suggested that it originates from a possible alteration of postnatal synaptic epigenesis (e.g., Karlsgodt *et al.*, 2008). Synapse elimination or "pruning" would be disrupted and would occur to an excessive degree in patients, placing them below a threshold for the presentation of psychotic symptoms. This disrupted epigenesis might possibly result from prenatal or perinatal insult or genetic disruptions at early stages of development or from a prolongation or excessive pruning process yielding too few functional synapses. Indeed, patients with schizophrenia when compared with controls show a reduction in cortical thickness and a reduction in dendritic spine density on prefrontal cortical pyramidal neurons (Glantz & Lewis, 2000) but without an accompanying reduction in the number of neurons (Selemon *et al.*, 1995).

Longitudinal MRI studies have further demonstrated a progressive decrease in brain volume in patients, and a surface contraction in the dorsal surfaces of the frontal lobe that may reflect an increased rate of synaptic pruning, resulting in excessive loss of long range neuronal connectivity (Sun *et al.*, 2008) in particular that assumed to contribute to the global neuronal workspace (Friston & Frith, 1995; Changeux & Dehaene, 2008; Chapter 23). Consistent with this interpretation, many of the potential risk genes identified thus far for schizophrenia are known to affect the processes of neuronal migration, myelination, neuronal integrity, and intracellular transport, all of which are plausibly implicated in the developmental path of the disorder (Sun *et al.*, 2008). A similar mechanism, though with a different timescale, may also apply to autism.

Synaptic epigenesis and the origins of culture

Brain epigenetic capacities to store stable representations of the outside world give human beings the opportunity to create an artificial world of cultural objects at the social level. The considerable increase in synapse numbers and multiple nested processes of synapse stabilization that take place postnatally in the human brain make possible the acquisition of spoken and written language (see Dehaene & Cohen, 2007), among many other social representations that can be stored in extracerebral memories (Changeux, 1994). But this epigenetic evolution has another consequence: the diversification of the cultures that human beings have developed throughout their recent history. In other words, the postnatal epigenetic evolution of brain connectivity opens the way to cultural evolution.

Representations, reward, and consciousness in the infant brain

Wittgenstein in his *Philosophical Investigations* in 1953 wondered how human beings can understand each other despite so many language differences. He suggested that the identity of the representations we build up in our mind (I would say in our brain) develops not only from shared "language games" or "experiments" that we play as infants, but also continuously throughout our lives as adults. For him the final criterion when somebody else possesses this representation is "what he says and what he does," in other words what use he makes in his current life of this particular representation. For the neurobiologist, Wittgenstein raises a serious issue. Will it ever be possible to identify the postulated "common element" in the brain of social partners who understand each other. We know, from the previous section, that the detailed connectivity of the brain may vary considerably from one individual to the other, even among identical twins.

A first reassuring statement comes from the mathematical formalization of the synapse selection

model. It is the theorem that within a developing network "the same afferent message may stabilize different arrangements of connections which nevertheless result in the same input-output relationship" (Changeux *et al.*, 1973; Changeux, 1983a,b). This may explain why the variability of the connectivity found between isogenic animals and *a fortiori* between nonisogenic ones may be behaviorally tolerable for the organism. This contrasts with Edelman's (1998) claim that because of such variability there should not be any code or representation in the stored traces of past experience. The way memories are stored in the brain is not expected to resemble those of our computer devices. But, some code nevertheless has to exist which allows reproducible behaviors and, even shared understanding within the social group (with a common semantic) between individuals possessing anatomically variable brains. What might constitute such codes?

It has been widely accepted since Hebb (1949) that the representations, images, ideas, or mental states that form in our brain spontaneously, or as a consequence of interactions with the outside world (including the sociocultural one), can be identified as the physical state created by the correlated (or coherent) transient and dynamic activity, both electrical and chemical, in a defined distributed population (or assembly) of neurons (see Gray *et al.*, 1992; Vaadia *et al.*, 1995; Engel & Singer, 2001). The graph of the neurons mobilized together with the frequency and coherence of the impulses flowing in the brain is anticipated to carry the "meaning" or, following de Saussure, the "*signifié*" (signified). How does a shared, neurally coded, *signifié* emerge? First, the commonality of meaning between different individuals may simply result from shared hardwired species-specific circuits of the infant brain. These are the "conspecs" of Johnson and Morton (1991) which, for instance, account in the infant brain for recognition of conspecific faces or speech sounds, in addition to elementary behaviors such as grasping and sucking. Second, very early on, the newborn learns features, for instance those unique to the mother's voice or face. These are the "conlerns" of Johnson and Morton. Within the framework of learning by selection (see Changeux, 1983a,b; Dehaene & Changeux, 1989), such learned *signifiés* would result from the selective stabilization of fleeting pre-representations spontaneously produced by the infant's brain.

The conjecture is that the brain would generate "crude," transient, and labile pre-representations – or hypotheses – which vary from one instant to the other. The infant brain would thus operate in a projective way (see Changeux, 1983b; 2004; Berthoz, 1997) constantly testing hypotheses about the outside world and later on its own inner world. Several plausible mechanisms may lead to the selection of the relevant pre-representation and to its storage as not only a stable synaptic and/or neuronal but also a semantic trace in the brain. A simple one (Dehaene & Changeux, 1989; Montague *et al.*, 1996; Schütz *et al.*, 1997; Edelman & Tononi, 2000; Fiorillo *et al.*, 2003, 2005) is based on the reward (or punishment) received from the outside world that would ultimately result in the release of reward substances, e.g., dopamine, acetylcholine, and serotonin. Accordingly, the efficacies of the synapses concerned would change if, for instance, the reward neurotransmitter reached the surface of the neurons at the moment that they were actively mobilized by the relevant pre-representation (or after a defined delay). Such a reading of time coincidence could be accomplished, for instance, by "multihead" molecules, named allosteric proteins, which include neurotransmitter receptors (see Dehaene & Changeux, 1989, 1991; Changeux & Edelstein, 1998, 2005). Those receptors are known to undergo slow regulatory transitions of potentiation and/or desensitization, which can be regulated by a variety of effectors, in particular phosphorylation (see Changeux & Edelstein, 1998, 2005). Yet, at this stage, their precise contribution to the storage of "meaningful" representations remains to be documented.

At a higher social level, what could be the actual mechanisms that result in a shared representation within the brains of different social partners? One possible mechanism in the case, for instance, of mother and child could involve a "shared attention" toward a common object or referent, which would accompany its designation by eye movements, gestures, and finally verbal utterances (De Boysson-Bardies, 1998). The *signifiant–signifié* link would progressively become established, first by selection of the *signifiés* via "cognitive games" which, subsequently, would develop in authentic "language games" between the child and his or her parents, peers, or teachers. Then, another distinction has to be made. The same referent resulting in the same neurally stored *signifié* may plausibly be viewed as shared by all members

of the human species, in other words be universal, but this is not to be anticipated for the arbitrary *signifiants*, which are known to vary from one language to another.

Recent brain imaging experiments support this point of view. First, in bilingual individuals who learned their second language after the age of 7, the first language production (or listening) systematically activated a similar set of areas, while the production (or listening) of the second language activated partially different and individually variable areas (Dehaene *et al.*, 1997; Kim *et al.*, 1997). Second, when subjects are presented with pictures and the corresponding words in letters, modality-specific activation occurs in different areas for words (left inferior parietal lobule) and for pictures (right middle occipital gyrus), while a semantic network common to both words and pictures extends, in a distributed manner, from the occipital and temporal cortex to the frontal lobe (Vandenberghe *et al.*, 1996; see also Gorno Tempini *et al.*, 1998). Moreover, different regions of the brain are mobilized by different semantic knowledge, such as knowledge about the body parts or about numbers (Le Clec'h *et al.*, 2000; Dehaene, 2007). Such distributed category-specific activation of the cerebral cortex appears largely reproducible from individual to individual at the macroscopic (centimetric scale) level. Yet, substantial variability is anticipated at the microscopic synaptic level. A plausible scheme which simultaneously accommodates macroscopic constancy, local variability, and universality of the semantic representation relies on the notion that it is not the precise anatomical connectivity between identifiable neurons which counts (as it does in the nervous system of *Caenorhabditis*). Rather a common "functional pattern" of connections, as opposed to a precise geometry, is selected by "shared" learning. The functional pattern of the connections as, for instance, a common menu of neurons sampled from a variety of brain areas would then neurally implement the semantic code. It is an important issue for further studies to define to what extent such neural anatomy of semantics displays (or not) analogies with the semantic networks postulated by linguists.

The newborn infant is an emotionally reactive sentient being. But he (or she) must still develop what makes human beings rational and self-conscious. These psychological functions progressively build up, in the following years, together with the memories of the semantic lexicon. The prefrontal cortex becomes accessible to delayed response tasks by 12 months (Diamond, 1991). A working memory and global workspace (see below) start to contribute to cognitive tasks, for instance those that are no longer automatic and demand a conscious mental effort. Global regulatory circuits that provide, in particular, reward and vigilance inputs (such as the mesocortical catecholaminergic neurons and cholinergic pathways) progressively develop their adult pattern of connections.

The neural bases of consciousness have been, in recent years, the subject of intense debates (Baars; Crick & Koch; Posner; Edelman; Llinas; Damasio; Dehaene & Changeux) (Fig. 1.4), but these are outside the scope of this review (see Searle, 2000; Tononi & Edelman 1998; Changeux & Dehaene 2008; Chapter 23). I will only mention recent modeling studies aimed at elaborating neural architectures able to successfully carry tasks demanding conscious effort. Accordingly, in simplified, formal terms, two main computational spaces can be distinguished in the brain (Dehaene *et al.*, 1998).

The first is a processing network composed of parallel, distributed, and functionally encapsulated processors ranging from primary (or even heteromodal) sensory processors and that include motor processors. A second is what can be referred to as a global workspace (Baars, 1989) consisting of a distributed set of cortical neurons characterized by their ability to receive impulses from, and send them back to, homologous neurons in other cortical areas by horizontal projections through long-range excitatory axons. Pyramidal cells from layers II and III, which are particularly abundant in dorsal, lateral, prefrontal and inferoparietal cortical structures, may be postulated to contribute to this workspace in a privileged manner.

The model posits (Dehaene *et al.*, 1998) that, in a "conscious" task, workspace neurons become spontaneously coactivated forming discrete, though variable, spatiotemporal patterns (some kinds of global prerepresentation) subject to modulation by vigilance signals and to selection by reward signals. Such workspace activation interconnects multiple brain processors (and suppresses the contribution of others) that would otherwise be mobilized (or extinguished) independently. As a consequence, the organism performs cognitive tasks requiring cross-modal processing such as the Stroop task (which involves the distinction between the color words and the color of the ink used to print the word). Such a scheme accounts in

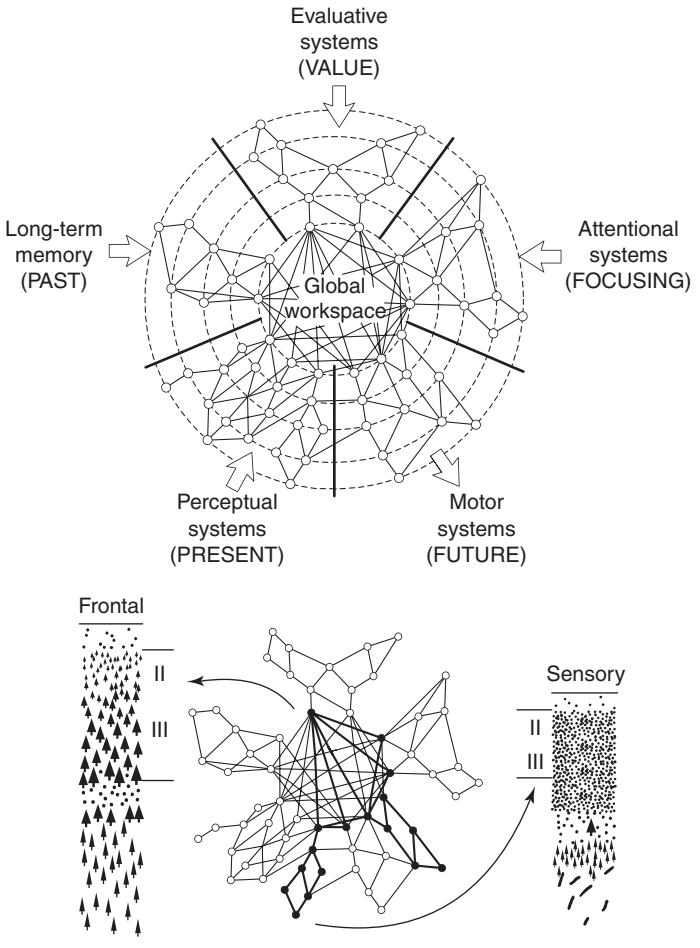

Fig. 1.4 A neuronal model of a global "conscious" workspace engaged in an effortful cognitive task. The global workspace is composed of distributed and heavily interconnected neurons with long-range axons originating, principally, from layers II and III from the cerebral cortex. The multiple processors (here 5) are modular and informationally encapsulated. Workspace neurons (abundant in frontal type cortex) are mobilized in effortful, multimodal, tasks for which the specialized processors do not suffice and regulate in a top-down manner the activity of specific processor neurons (from Dehaene *et al*., 1998).

particular for brain imaging data. In particular, it predicts the active, although transient, mobilization of the dorsolateral prefrontal cortex and anterior cingulate areas, which are known to contain a very high proportion of layer II and III long-range excitatory neurons.

The model is still at a primitive stage of formalization and has shortcomings. It should integrate, in the future, the connection to the workspace of self-representations that might allow the simulated organism to reflect on its own internal processes. Longer-term research should also include the more elaborate dynamic organization of nested workspace units (Dehaene & Changeux, 1997) and auto-evaluation loops (Dehaene & Changeux, 1991). Modelization of the linguistic proposition and of rational reasoning in terms of neural networks is still a far-fetched enterprise. On the other hand, brain imaging and event-related potential techniques allow the determination of neural events during which, in agreement with the model, brain activity correlates with conscious versus nonconscious processing (Sergent *et al*., 2005; Del Cul *et al*., 2007; Haynes *et al*., 2007). In any case, imaging of the infant brain and of its postnatal development is anticipated to bring essential information on the development of the neural bases of higher brain functions including thought and consciousness (see Chapter 23).

Conclusions

These reflections on the origins of the human brain are still fragmentary and rather speculative. They nevertheless illustrate the urgent need for adequate theories (or models) that establish neurally plausible links between the anatomy and molecular biology, the physiology and biochemistry and the actual behavior with the world as well as the "tacit" mental states that arise in the infant brain.

Aware that the whole sequence of the human genome is known, the overall philosophy of the neurosciences is anticipated to shift from a strictly "reductionist" point of view to a "reconstructionist" approach. Knowing all the genes that serve as building blocks of the human being, the emphasis will be to understand the molecular and cellular networks of interaction which yield the so-called "complexity" of the human brain. The task is immense and may not yield a unique answer. The "epigenetic" internalization of the personal history of each individual, together with the social and cultural environment in which he or she developed, raises tremendous problems of analysis and formalization. In any case, the brain of each human individual will meld, in a singular manner, multiple nested evolutions by variation selection: the genetic evolution of the species, together with the many epigenetic evolutions of the experiences and memory traces stored within the brain and also outside the brain as a cultural heritage of civilizational history.

Still promising, but increasingly difficult, will thus be the scientific understanding (in neuronal terms) of particularly elaborate cognitive functions (Shallice, 1998) such as those involved in esthetic judgment (see Changeux, 1994) and the genesis of moral norms. These functions include, for instance, the attribution of mental state to others (theory of mind), the so-called moral emotions such as sympathy and/or violence inhibition and, of course, the ability to integrate past memories and present information into a decision-making process leading to action in a social world (see Changeux & Ricoeur, 1998; de Vignemont & Singer, 2006 for references). Yet, one should not forget that all these elaborate cognitive processes become established in the human brain under conditions in which the infant and the child are never in social isolation but, on the contrary, are constantly interacting with other human beings. If a spontaneous tendency exists for the biologist to consider the human brain as an independent and autonomous object of science, this is a misleading endeavor. First of all, during postnatal development, interaction with parents, playmates, and classmates leads to the spontaneous acquisition of the maternal oral language. Subsequently, but then with considerable effort, the child acquires written language. The deep traces left in the human brain by alphabetization underlines the vast importance of early training (Castro Caldas *et al.*, 1998; Dehaene, 2007) in the connectivity and performance of the adult brain.

Pedagogy, according to Premack, is a unique attribute of human beings, and the early social environment in which the child is embedded also marks each infant brain by the particular culture and thus the history of the social community to which his or her parents belong. This constitutes, by its diversity, an unlimited source of innovation (Levi-Strauss, 1961). But, on a world scale, it may also be a source of ethnic conflict. In between the universality of the human species and the diversity of cultures, the brain makes the synthesis. It is a wish of neuroscientists that a better understanding of our brain will help to reconcile these opposing tensions.

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Section 1
Chapter
2

Making of the brain

The molecular basis of central nervous system development

Ola Hermanson and Urban Lendahl

Introduction

The aim of this chapter is to discuss how the central nervous system (CNS) is generated during development, and the rapid progress that is made in deciphering the underlying molecular programs for different steps in this process. Particular emphasis will be placed on the discussion of genes that are directly relevant for human CNS malformations.

Morphology of the developing CNS

In humans and other vertebrates, the early aspects of nervous system development proceed through a set of stereotypical intermediate steps. The first signs of the developing nervous system appear on the dorsal side of the gastrula-stage embryo where, in a process called neural induction, the mesoderm induces the overlying ectoderm to form the neural plate or neuroectoderm. The exact mechanism by which the mesoderm influences the ectoderm-to-neuroectoderm transition is still not understood, and hypotheses range from those suggesting the neuroectoderm develops by default mechanisms to those of a more active, instructive role for the mesoderm (see below).

After neuroectoderm formation, the neural plate bends along the rostrocaudal body axis to form the neural groove, and this change in morphology is accomplished by forces exerted through both the neural plate cells and the surrounding epidermis (Fig. 2.1). The notochord induces these cells to decrease in height and to become wedge shaped, giving rise to the neural groove (van Straaten *et al.*, 1988; Smith & Schoenwolf, 1989). Shortly thereafter, the edges of the neural plate thicken and move upward to form the neural folds. The cells in the regions near the ectodermal boundaries increase in height and become wedge shaped as well. At the same time, the surface ectoderm pushes itself towards the center of the embryo, further bending the neural plate (Alvarez & Schoenwolf, 1992). Eventually, the neural folds adhere to each other and the cells from the two folds merge, closing the neural groove to form the neural tube (Fig. 2.1). Therefore, formation of the neural tube is largely a result of changes in cell shape and cell–cell interactions, and cellular proliferation contributes only to a minor extent. The cells in the junctional region of the neural folds and later at the dorsal aspects of the neural tube form the neural crest cells, which migrate to other regions and give rise to the peripheral nervous system, the pigment cells of the skin, and several other cell types. The delamination of the neural crest cells from the neural groove and tube and their subsequent migration is associated with robust morphological changes in the cells, referred to as epithelial-to-mesenchymal transitions (Jeffery, 2007).

In humans and other mammals, neural tube closure is initiated at several positions along the anteroposterior axis. Failure of the neural tube to close results in neural tube defects. Lack of closure of the rostral neural pore leads to anencephaly, while failure of closure of the caudal pore results in spina bifida. The frequency of neural tube defects (NTDs) is affected both by chemical compounds such as the antiepileptic drug valproic acid and by genetic determinants, i.e., NTDs are more common in certain human populations. After congenital heart defects, NTDs are the most common birth defect, with a birth incidence of approximately 1/1000 in many populations (Detrait *et al.*, 2005). Support for a genetic component in NTDs comes from associations with genetic syndromes, such as Meckel syndrome, and with chromosome 13 and 18 trisomies. In mouse models for NTDs, more than 100 genes have been directly or indirectly implicated in NTDs, but the list of genes firmly associated with human NTD is considerably shorter. Mutations in the *PAX3* gene (see below) can, however, cause NTDs and also result in Waardenburg

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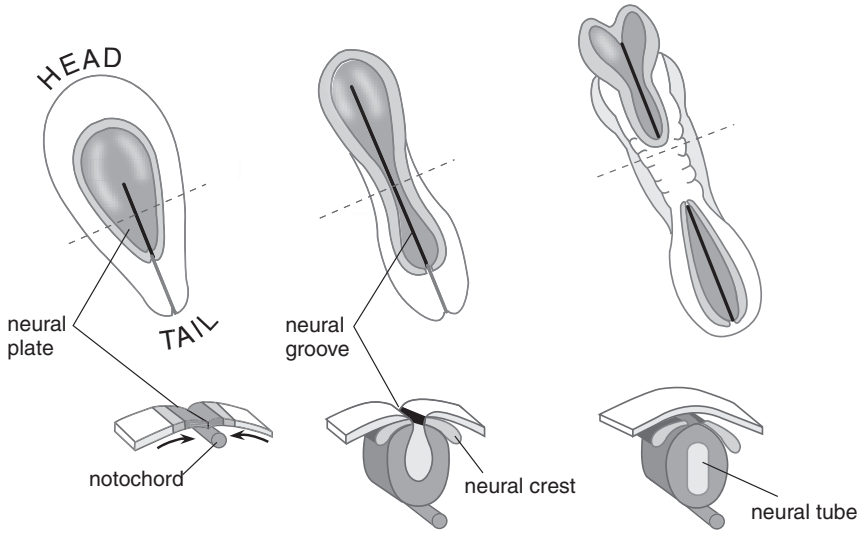

Fig. 2.1 Schematic depiction of the early aspects of development of the central nervous system (CNS), seen in a cross-section of a mammalian embryo. The first sign of nervous system formation is the appearance of the neural plate on the dorsal aspect of the embryo. The notochord is located beneath the neural plate in the midline. The neural plate folds and forms the neural groove. The neural folds fuse to form the neural tube. The neural tube is then pinched-off from the dorsal ectoderm, both ends of which fuse to form the epidermis.

syndrome, which affects neural crest derivatives (Baldwin *et al.*, 1992). Similarly, mutations in the human *TWIST* gene result in the Sa ethre–Chotzen syndrome, an autosomal dominant craniosynostosis disorder (Howard *et al.*, 1997). It is becoming increasingly clear that folic acid supplementation can to a considerable extent reduce the incidence of spina bifida, but the underlying molecular mechanisms are poorly understood (Mitchell *et al.*, 2004).

The newly closed neural tube is initially composed of a single layer of highly proliferative cells. Cell lineage experiments, both in vivo and in primary culture, reveal that these proliferating cells can, in addition to self-renewal, give rise to all major classes of cell types in the CNS, i.e., both neurons and glial cells (Merkle & Alvarez-Buylla, 2006). The CNS stem/progenitor cells are organized as a pseudostratified columnar epithelium. The neuroectodermal cells extend from the inner, ventricular, to the outer, pial, side of the neural tube (Fig. 2.2). They typically have a bipolar morphology and undergo complex morphogenetic changes called interkinetic movements as they progress through the cell cycle. The elongated nucleus lies in the luminal half of the ventricular zone during the G1 phase of the cell cycle, and migrates into the pial half during the S phase, when replication of DNA occurs (Fig. 2.2). The nucleus then translocates back again near the ventricular surface in the G2 phase. At the same time the cells detach from the pial side and the pial processes collapse; the cells then divide on the ventricular surface of the neural tube. After completion of mitosis, the pial process reextends toward the basal surface in both daughter cells or, alternatively, one cell differentiates into a neuron and migrates out from the columnar neuroepithelium (see below) (Sauer, 1935; for a review, see Jacobson, 1991).

After proliferation of this initial, founding population of cells, it is important for the cells to achieve the correct balance between maintaining a pool of stem/progenitor cells for generation of more cells at later time points and the production of specialized cell types: initially neurons and later glial cells. This is in part accomplished by creating and maintaining a fine balance between symmetrical and asymmetrical cell divisions in the CNS stem/progenitor cells, such that an increasing proportion of differentiated cells is generated over time (Kriegstein *et al.*, 2006). Symmetrical cell division generally generates two new stem/progenitor cells, whereas asymmetrical cell division generates one stem cell and one cell that can undergo differentiation. The molecular machinery controlling asymmetrical cell division has been extensively studied in

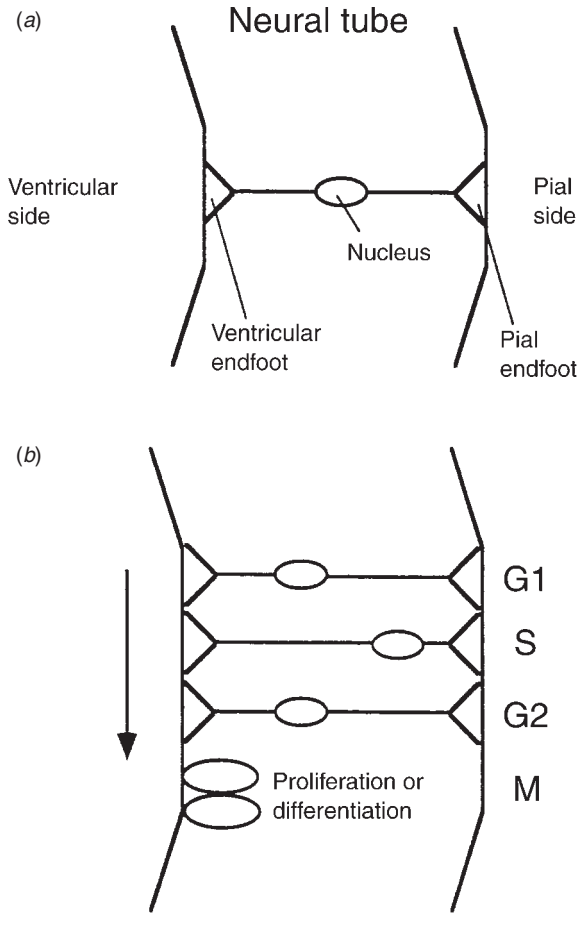

Fig. 2.2 Morphology of the early neural tube. (*a*) Schematic representation of the early neural tube, which at this stage consists of a single-cell layer organized as columnar neuroepithelium. The bipolar neuroepithelial cells are attached at the ventricular and pial sides by structures called endfeet. (*b*) The process of interkinetic movements, in which the nucleus of the neuroepithelial cell relocates during the cell cycle. During the G phase, the nucleus is located closer to the ventricular side, whereas during the S phase it is located closer to the pial side. During the M phase, the cell detaches from the pial side and undergoes cell division close to the ventricular side. The cell progeny can then remain as undifferentiated cells in the neural tube, or can migrate from the neuroepithelium to differentiate into a neuron. (Reprinted with permission from Frisén *et al.*, 1998.)

Drosophila, and several components, such as Numb, Prospero, and atypical protein kinase C, are conserved in mammals (Knoblich, 2008). The orientation of the mitotic cleavage plane is important for the balance between maintaining stem cells and generating differentiated progeny. The orientation of the cleavage plane is initially perpendicular to the ventricular surface and then changes to become more in parallel with the ventricular surface. This results in cells closest to the ventricular surface retaining their stem cell properties, whereas the cells further away migrate and undergo neuronal differentiation.

In conjunction with the increasing number of cells, the CNS converts into a more complex, multilayered structure, in which the CNS stem/progenitor cells are confined to the inner, ventricular layer, and the newly formed daughter cells migrate outwards to specified layers of the developing cortical plate and spinal cord. During this migratory phase, the young postmitotic neurons are guided by specialized cells, the radial glial cells. The radial glial cells are elongated cells that traverse the thickened neural tube from the inner ventricular to the outer pial side. Radial glial cells are anchored by endfeet to both surfaces of the neural tube (Fig. 2.2), and they retain their extended morphology throughout the cell cycle. Radial glial cells are important in the neurogenic process, and it has been postulated that because of their guiding role for the young migrating neurons, they are important for the columnar organization of neurons in the cortex (Rakic, 1988). It is, however, becoming increasingly clear that neurons are not only positioned by migration along radial glial cells, but that many neurons migrate in a tangential rather than a columnar fashion. This is particularly the case for cortical interneurons, which migrate tangentially from their origins in the ganglionic eminences (Wonders & Anderson, 2005). More recently, radial glial cells have been shown not only to serve as guide posts for migrating neurons, but also actually to function as an additional population of CNS stem/progenitor cells which can give rise not only to astrocytes, but also to neurons (Fricker-Gates, 2006).

Early brain development results in a layered organization of cells in the developing cortex. As a result of the sustained proliferation of CNS stem/progenitor cells, daughter cells migrate from the ventricular zone to form the first neuronal layer, the preplate, which forms on top of the ventricular zone. Next, between the ventricular zone and the preplate, a second layer of proliferative cells, the subventricular zone, is formed. The preplate then splits into an upper marginal zone and a deeper subplate, and a cortical plate is produced between these two layers (Kriegstein *et al.*, 2006). Those neurons that develop first form the layers closest to the ventricular zone and neurons that develop subsequently migrate through these regions to form the more superficial layers of the cerebral cortex. Formation of the mammalian neocortex therefore follows an "inside-out" gradient of development (Rakic,

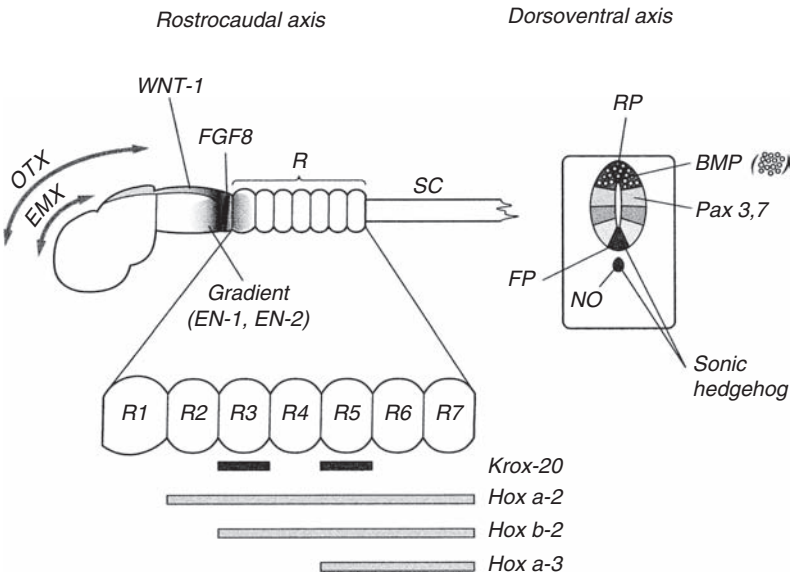

Fig. 2.3 Schematic representation of a mammalian embryo seen along the rostrocaudal axis (left) or along the dorsoventral axis (right). Some genes important for the patterning along these two axes are depicted in the figure. These genes are also discussed in the text. T, telencephalon; D, diencephalon; M, midbrain; R, rhombomeres; SC, spinal cord; RP, roof plate; NT, neural tube; FP, floor plate; NO, notochord.

1974). Exceptions to this general pattern of cortical development are found in the cerebellum and the hippocampus, where cohorts of progenitor cells migrate from the ventricular zone, and later form neurons and glial cells.

The cortex in humans and other primates is highly folded (gyrencephalic) in contrast with the cortex of other mammals, for example the cortex in rodents, which is more smooth (lissencephalic). The reason behind this evolutionary transition is still being debated, but it is of note that disturbances in the formation of the various layers in the human cortex lead to CNS malformations that in some cases revert organization to a more lissencephalic state (Lian & Sheen, 2006). Mutations in four genes (*ASPM*, *CDK5RAP2*, *CENPJ*, and microcephalin) have been associated with microcephaly vera, and these genes are all expressed at the proliferative stages. Periventricular heterotopia, in which neuronal clusters are ectopically located along the walls of the lateral ventricles, is associated with filamin-A and *ARFGEF2* mutations, which both affect the actin cytoskeleton. The X-linked genetic disease lissencephaly, or double cortex, causes a disruption of the structure of the six-layered cortex, and loss of sulci and gyri in the brain, i.e., a conversion from a gyrencephalic to a lissencephalic phenotype. In a large proportion of cases, lissencephaly is caused by loss-of-function mutations in two genes: lissencephaly1 and doublecortin. The proteins encoded by these two genes are involved in microtubule organization (Lian & Sheen, 2006).

Patterning of the early CNS

At the same time as the cytoarchitecture of the future brain and spinal cord is being established, the different CNS regions gradually acquire spatial characteristics, which are reflected in the establishment of domains with unique gene expression patterns. This is important to provide unique cellular identities along both the rostrocaudal and the dorsoventral body axes. Some of the genes that are discussed below are summarized in Fig. 2.3.

The rostrocaudal axis

In mammals, it is clear that a first crude pattern along the rostrocaudal axis of the emerging neuroectoderm is already established during neural induction in the gastrula-stage embryo. It appears that the prechordal mesoderm, which is the rostralmost continuation of the notochord, specifies rostral, head-specific features, whereas the notochord itself induces only more caudal neural tissue (for reviews, see Lumsden & Krumlauf, 1996; Rubenstein & Beachy, 1998). Along the rostrocaudal axis, patterning is most conspicuous for the expression of various transcription factors, in particular for members of the clustered homeobox-containing genes of the Hox family (capitalized as *HOX* genes in humans). The four *Hox* gene clusters (A–D) in the mouse and human genome, consisting of 39 genes in total, are homologous to the homeotic gene complex (HOM-C) in *Drosophila*, which controls parasegment identity in

the fly. These gene clusters show remarkable evolutionary conservation when flies and mammals are compared, in that structurally equivalent genes are arranged in the same order on the respective chromosomes and show similar expression patterns along the rostrocaudal axis of the embryo. It therefore seems likely that the four mammalian *Hox* complexes have evolved from the fly complex through gene duplications and deletions (Hunt & Krumlauf, 1992). *Hox* genes are expressed throughout the developing neural tube from the anterior boundary of the hindbrain to the tail, including neural crest, paraxial mesoderm, surface ectoderm, and their derivatives. Strikingly, the order in which the genes of each cluster are positioned along the respective chromosomes is largely colinear with the order of their expression domains along the rostrocaudal axis of the embryo. The role of *Hox* gene expression has been best studied in the rhomberic patterning of the chick and mouse hindbrain and at somite boundaries further caudally in the embryo (Wilkinson *et al*., 1989; Kessel & Gruss, 1991) (for a review, see Mark *et al*., 1997).

The highly defined pattern of expression of the murine *Hox* genes has led to the formulation of the "Hox code" hypothesis, whereby particular combinations of *Hox* genes would specify the segmental identity along the rostrocaudal axis. The notion of a Hox code is supported by both ectopic expression and gene targeting experiments in the mouse, i.e., loss of function of a *Hox* gene generally results in anteriorization of the region in which this gene is normally expressed, and conversely, ectopic expression leads to posteriorization (for a review, see Krumlauf, 1994).

The teratogenic effects of retinoic acid (RA) can at least partly be explained by deregulation of *Hox* gene expression. Exposure of mouse embryos in utero to excess doses of RA results in posteriorization of the regional fate in the developing hindbrain, i.e., anterior rhombomeres assume the identity of more posterior rhombomeres (Marshall *et al*., 1992). This is caused by the anteriorization of expression of *Hox* genes that are normally expressed only in more posterior domains along the rostrocaudal axis. A similar posteriorizing fate effect is observed when *Hox* genes are expressed ectopically in the hindbrain or after injection of a constitutively active RA receptor (RAR) into the embryo (Zhang *et al*., 1994; Blumberg *et al*., 1997). At the molecular level, these results are consistent with a direct induction of *Hox* gene expression by binding of ligand/RAR complexes to RA response elements in the upstream regulatory regions of some *Hox* genes (Studer *et al*., 1994; Gould *et al*., 1998).

Given the highly conserved pattern of *Hox* gene expression, it is of interest to establish the upstream regulators of *Hox* gene expression. It is becoming increasingly clear that epigenetic regulation of the large chromosomal regions encompassing *Hox* genes plays an important role, and epigenetic regulators, such as the Polycomb and trithorax genes, have been shown to be important for setting up expression domains and the temporal order of *Hox* gene expression (Kiefer, 2007). Other transcription factors, such as Krox-20 – a zinc finger transcription factor, can also influence *Hox* expression. Krox-20 is expressed in two stripes of the neural plate that will become rhombomeres 3 and 5 (Wilkinson *et al*., 1989), and can directly regulate the expression of Hoxa-2 and Hoxb-2 in these two rhombomeres (Nonchev *et al*., 1996). Targeted disruption of the *Krox-20* gene results in embryos lacking rhombomeres 3 and 5 and showing a partial fusion of rhombomeres 2, 4, and 6 in the hindbrain region (Schneider-Maunoury *et al*., 1993). Long-range signaling from the isthmic constriction, a region located close to the junction of the mesencephalon (midbrain) and rhombencephalon (hindbrain), may also play a role in regulating *Hox* gene expression. Signaling from this region is mainly controlled by a secreted molecule, fibroblast growth factor 8 (FGF8). FGF8 is first expressed in the axial mesoderm underlying the presumptive isthmic region of the neural plate and later in a transverse stripe within the isthmus (Crossley & Martin, 1995).

In addition to the *Hox* genes, several other factors are important for rostrocaudal patterning. Wnt signaling (Wnt-1) and the transcription factor En-2 play critical roles in midbrain patterning. Wnt-1 and En-2 (Crossley *et al*., 1996; Lee, 1997) are homologs to the *Drosophila* genes Wingless and Engrailed, respectively. Wnt-1 is expressed in the neural plate in a region corresponding to the future midbrain and later in a ring of cells lying immediately anterior to the FGF8-secreting cells of the isthmus. Secretion of Wnt-1 is required for maintenance but not induction of *En-1* and *En-2* gene expression (McMahon *et al*., 1992), which is critically involved in the specification of rostrocaudal polarity in the midbrain/hindbrain junctional region.

As members of the *Hox* gene family are not expressed in the rostralmost neural tube, it is conceivable that related factors may fill their niche there. In the

mouse embryo, the homeobox-containing transcription factors Emx-1, Emx-2, Otx-1, and Otx-2 are expressed in a similar nested array in the forebrain and midbrain region, as are the *Hox* genes in more posterior regions (Shimamura & Rubenstein, 1997). Evidence for a role of the *Otx* genes in brain development comes from gene targeting experiments, as *Otx-2* null mutants lack forebrain and midbrain structures, consistent with an anterior patterning function during early neurulation stages (Acampora *et al.*, 1995). The developmental distortions after targeted mutation of the two *Emx* genes in mice are restricted to the dorsal telencephalon (the rostralmost part of the forebrain), corresponding to their restricted expression domain (Boncinelli *et al.*, 1993; Yoshida *et al.*, 1997). In keeping with this, mutations in the human *EMX-2* gene lead to severe defects in the structure of the cerebral cortex, a condition that is known as schizencephaly (Faiella *et al.*, 1997).

The distribution of two morphogens, RA and FGF, is also important for proper rostrocaudal differentiation (Diez del Corral & Storey, 2004). FGF is important for the more caudal aspects of differentiation, and is thus more highly expressed caudally. The converse is true for RA, which is more abundant in the rostral parts. It should, however, be noted that FGF and/or RA also play a role in differentiation in more rostrally located signaling centers in the brain, such as the isthmus.

The dorsoventral axis

The dorsoventral patterning events are best understood in the developing spinal cord, and will therefore be discussed in most detail for this region, although patterning appears to be similar in the more rostral regions of the CNS. Generation of dorsal cell types is controlled by signals from the dorsal (epidermal) ectoderm at the lateral borders of the neural plate, whereas generation of ventral cell types is triggered by signals from the notochord and prechordal mesoderm underlying the midline of the neural plate. Three secreted signaling molecules play crucial roles in the initiation of dorsoventral identity specification in the neural tube: bone morphogenetic proteins (BMPs), Wnt and Sonic hedgehog (Shh) (Ulloa & Briscoe, 2007). BMPs are members of the transforming growth factor-β (TGF-β) superfamily and are produced predominantly on the dorsal side by cells located at the boundary between the neuroectoderm of the neural plate and the lateral epidermal ectoderm (for a review, see Mehler *et al.*, 1997). After neural tube closure, several BMPs are expressed in overlapping domains along the dorsal midline ectoderm (Ulloa & Briscoe, 2007). BMPs induce dorsal cell types in the neural tube by promoting the expression of specific transcription factor genes, such as *Pax* and *Msx*, and by antagonizing the activity of Shh from the ventral side (see below). Similarly, Wnts, which are palmitoylated glycoproteins, are produced at the dorsal side and promote dorsal identities. In particular, Wnt-1 and Wnt-3a appear to be produced dorsally, and loss of either Wnt-1 or Wnt-3a results in reduction of cells typical of the dorsal neural tube. As discussed above, Wnt-1 also has a role in rostrocaudal patterning in the mid-hindbrain formation process.

Shh, together with Indian and Desert Hedgehog, constitute the three members of the vertebrate Hedgehog family of secreted glycoproteins. They were originally identified by their structural similarity to the *Drosophila* segment polarity gene Hedgehog (for a review, see Hammerschmidt *et al.*, 1997). Shh is initially expressed in the notochord and prechordal plate, and later also in floor plate cells lying at the ventral midline of the neural tube (Ericson *et al.*, 1996). Shh may be secreted from both notochord and floor plate cells and subsequently diffuses from this site, thereby establishing a concentration gradient within the ventral neural tube. Shh has a key role in specifying neuronal subtype identity. Ectopic expression of Shh can induce ventral cell types, and mouse Shh null mutants show a loss of ventral structures throughout the CNS (Echelard *et al.*, 1993; Chiang *et al.*, 1996). Shh appears to act through repression and induction of specific transcription factors in a concentration-dependent manner along the dorsoventral axis of the neural tube (for a review, see Tanabe & Jessell, 1996). Shh is synthesized as a larger precursor that undergoes autoproteolytic cleavage to generate a biologically active amino-terminal fragment (Porter *et al.*, 1995). During the autocatalytic processing of Shh, cholesterol is covalently attached to the carboxy-terminus of the new amino-terminal fragment. Shh acts via two transmembrane receptors at the signal-receiving cell. One of the transmembrane proteins, Patched, binds Shh and this binding initiates signaling from the second protein, Smoothened. Downstream of Smoothened, intracellular signaling pathways regulate the activity of Gli transcription factors via proteolytic processing of the Gli proteins (Ruiz i Altaba *et al.*, 2003).

A consequence of the Shh concentration gradient on the ventral side of the neural tube is the establishment of precise dorsoventral expression domains of a set of homeodomain-containing transcription factors (Briscoe & Ericson, 2001). Five domains are generated, which (from the ventral side) give rise to V0, V1, V2, MN, and V3 neurons. The transcription factors are of two types, class I and II proteins, and class I and II factors are cross-repressive, which is important to generate and maintain the sharp expression boundaries between each domain. In the dorsal half of the neural tube, proteins of the Pax family are important for patterning. The *Pax3* and *Pax7* genes, for example, are expressed initially along the mediolateral region of the neuroectoderm, but are rapidly repressed in the medial neural plate by a Shh-mediated signal. After neural tube closure, the Pax3 and Pax7 expression domain is restricted to proliferating cells in the dorsal neural tube (Liem *et al.*, 1995; Ericson *et al.*, 1996). Notably, mutations in the human *PAX3* gene have, as discussed above, been associated with spina bifida.

Several of the factors setting the dorsoventral and rostrocaudal axes of the neural tube are also important for forebrain regionalization. BMP4 and Wnt-3a are expressed in the dorsalmost telencephalon and are required for the correct development of dorsal structures such as the choroid plexus and the hippocampal formation (Sur & Rubenstein, 2005). FGFs, including FGF8, exert counteracting "ventral" signals that contribute to the right organization and cell number. Shh signaling is also important in forebrain development (for a review, see Rubenstein & Beachy, 1998). The defects observed in Shh knockout mice include absence of ventral forebrain structures and a failure to subdivide the eye field, which results in the formation of a cyclopic eye, while the remainder of the forebrain develops as a single, undivided vesicle (Chiang *et al.*, 1996). A similar phenotype is observed in the most severe form of human holoprosencephaly (for a review, see Ming & Muenke, 1998) and, indeed, mutations in the coding region of the human *SHH* gene are associated with an autosomal dominant form of human holoprosencephaly in several pedigrees (Roessler *et al.*, 1996). Furthermore, mutations in the carboxy-terminal domain of the Shh protein, responsible for the autocatalytic cleavage and cholesterol transfer to the amino-terminal fragment, have been associated with several cases of familial and sporadic human holoprosencephaly (Roessler *et al.*, 1997). Moreover, perturbations of the cholesterol metabolism in pregnant rats, ewes, and in humans all lead to development of holoprosencephalic phenotypes in the offspring, suggesting that these perturbations affect the Shh signaling pathway (for a review, see Rubenstein & Beachy, 1998). Mutations in several other loci are also associated with human holoprosencephaly and other developmental disorders, and some of those affect genes located in the Shh downstream signaling pathway (Roessler & Muenke, 2003).

Molecular programs controlling differentiation of CNS stem cells

In addition to the patterning of regions in the developing CNS that provides cells with the necessary spatial information for acquiring the correct identity, there are molecular programs that more generally control the balance between maintenance of cells in the stem/progenitor cell state and the promotion of differentiation to neurons. Two such programs are Notch signaling and the family of Sox transcription factors. Notch signaling is an evolutionarily conserved signaling mechanism for cell–cell communication. Ligand activation leads to cleavage of the transmembrane Notch receptor, which liberates the intracellular domain of the receptor. The intracellular domain translocates to the cell nucleus, where it serves as a transcriptional activator (Louvi & Artavanis-Tsakonas, 2006). In early CNS development, Notch signaling blocks neuronal differentiation, partly via activation of Hes transcription factors, which repress expression of differentiation-promoting factors such as the neurogenin transcription factors. Members of the Sox protein family can have functions that promote both stem cell maintenance and differentiation (Wegner & Stolt, 2005). While Sox1, 2, and 3 maintain the stem cell fate, Sox21 has the opposite role, and promotes neuronal differentiation. Sox9 is critical for promoting glial rather than neuronal fates, whereas Sox10 is required for oligodendrocyte differentiation (Wegner & Stolt, 2005).

Many additional factors and signaling pathways act in parallel with or cross-talk with notch signaling and Sox factors to control CNS stem cell proliferation and lineage decision. Such additional factors include transcription factors downstream of cytokine or BMP signaling, e.g., signal transducers and activators of transcription (STATs) and Smad factors (Miller & Gauthier, 2007). In general, increased STAT or Smad

activity promotes astroglial differentiation of CNS stem cells. Certain homeodomain transcription factors, including Pax6, Dlx, and POU factors, are necessary for normal neurogenesis. The zinc finger factor REST (RE1 silencing transcription factor)/NRSF (neuron-restrictive silencing factor) was first shown to be a repressor of neuronal genes in non-neuronal cells, and is also important to maintain CNS stem cells in an undifferentiated state.

Many studies have demonstrated important roles for transcription factors of the basic-helix-loop-helix (bHLH) family in regulating lineage decision. Hes factors (mentioned above) are most often associated with repression of neurogenesis. Neurogenins and closely related factors, such as Mash1 and Math1, promote neurogenesis, and after the initial decision, bHLH factors such as NeuroD1 control the progression of neuronal differentiation. Members of another subclass of bHLH transcription factors, Olig, are required for oligodendrocyte differentiation, and gene deletion of Olig2 results in increased astrocyte differentiation. In addition to signaling pathways and DNA-binding transcription factors, transcriptional coregulators such as *SMARCA4* (*BRG1*), *CREBBP* (*CBP*), and *NCoR1* are essential for control of CNS stem cell proliferation, characteristics, and appropriate differentiation (Miller & Gauthier, 2007). Coregulators are factors that do not bind DNA themselves but are recruited to the chromatin by DNA-binding transcription factors. These coregulators either interact with chromatin-modifying proteins or themselves exert chromatin-modifying activity.

Chromatin modifications in CNS development and disorders

In addition to the effects of individual transcription factors acting at various stages of CNS development, there is an emerging view that mechanisms that more globally affect gene transcription also are important for CNS development and in the genesis of many neurodevelopmental disorders. Such epigenetic changes (epigenetics is here defined as alterations in the gene expression or regulation thereof that do not involve changes in the DNA sequence) may, for example, provide the molecular basis of neurodevelopmental disorders such as the Rett syndrome, Prader–Willi syndrome, and Angelman syndrome, and certain psychiatric disorders have been linked to factors that are regulated by or regulate chromatin structure (Kiefer, 2007). Various modifications of DNA itself or of the histone proteins are important epigenetic mechanisms for regulation of the degree of compaction of nuclear DNA, which is wrapped around histones in the form of chromatin (Kiefer, 2007). The most studied chromatin modifications are DNA methylation and histone modifications, in the form of methylation, ubiquitination, and acetylation. Many of these modifications facilitate or repress transcription of the genes by affecting the local structure or "nanoarchitecture" of the gene loci, and a subset of modifications can also affect higher-order chromatin structure. Histone 3 and 4 (H3 and H4) are particularly frequent targets for modifications, in particular in the amino-terminal parts of these histones, the so-called "histone tails" (Kiefer, 2007).

Acetylation of histones is an important means to control the chromatin state (Smith, 2008). In the mid 1990s, it was shown that transcriptional activators and repressors in yeast could be linked to histone acetylation and deacetylase activity, respectively. Histones are predominantly acetylated on lysine (K) residues. Deacetylated histones, with "naked" lysine residues, are positively charged and avidly bind the negatively charged DNA, resulting in a tight chromatin structure and presumed repression of transcription in particular when located in a promoter. In contrast, acetylation of lysines results in a neutralized histone charge and thereby a more open chromatin structure, presumably facilitating transcription initiation. Lysine 9 on histone H3 (H3K9) is a frequent acetylation target for transcriptional activators such as the histone acetyl transferases (HATs) CBP, pCAF and p300, whereas transcriptional repressors exert histone deacetylase (HDAC) activity on the same lysine residue (Shi *et al.*, 2003). It is, however, of note that targets for HATs and HDACs are not restricted to histones, but can include many types of protein, including transcription factors (Smith, 2008).

HDACs are relatively well defined and divided in three major classes – classes I, II, and III (also referred to as sirtuins) – and one minor class, IV (Yang & Seto, 2008). In contrast, the various HATs are more heterogeneous in nature, and di cult to subcategorize into distinct classes. Mutations in the HAT CBP have been shown to cause Rubinstein–Taybi syndrome, a well-defined syndrome characterized by mental retardation and physical abnormalities (Petrij *et al.*, 1995). Furthermore, it has been reported that mutations in the closely related factor p300 cause Rubinstein–Taybi-like symptoms, including mental retardation

supposedly due to aberrant CNS development (Abel & Zukin, 2008). HDACs have been indirectly implicated in neurodevelopmental disorders and psychiatric disease. This is not based on reports directly linking HDAC mutations to disease, but on observations that inhibitors of class I, II, and IV HDACs, such as valproic acid, can cause malformations. For example, the majority of the surviving offspring of mothers who have taken valproic acid develop autistic features (Abel & Zukin, 2008).

Histone methylation has, like histone acetylation, proven to be essential for proper CNS development, and dysregulation of factors regulating histone methylation have been linked to disease (Feng *et al*., 2007). Methylation occurs on lysine and arginine residues. Histone methylation prevents acetylation and demethylation is thereby a prerequisite for acetylation. In contrast to acetylation that only exists as monoacetylation, methylation can occur also as mono-, di-, or trimethylation. Histone methylation is mediated by histone methyl transferases (HMTs). Histone methylation was long considered to be an irreversible mark, but in recent years a series of histone demethylases have been discovered, including LSD1 (KDM1) and a large family of proteins containing the so-called Jumonji domains (JMJD or KDM factors) (Takeuchi *et al*., 2006). Like histone acetylation, histone methylation is a dynamic and regulated process. The discovery of histone demethylases revealed close links to early and late CNS development. Methylation of lysine 27 on histone H3 (H3K27) is a critical mark of repression associated with Polycomb activity and undifferentiated progenitor state. The factor JMJD3 was shown to be a H3K27 demethylase opposing the activity of the Polycomb complexes (Lee *et al*., 2007), and essential for correct regulation of *Hox* genes and rostrocaudal axis development. JMJD3 expression is upregulated by retinoic acid and repressed by the RAR-associated repressor NCoR2, and later in development seems crucial for correct differentiation of γ-aminobutyric acid (GABA)ergic progenitors (Jepsen *et al*., 2007).

In addition to modifications of histones, DNA can also be modified, and most notably by DNA methylation. DNA is methylated primarily on CpG dinucleotides, and methylation is carried out by a family of DNA methylases comprising Dnmt1, Dnmt3a, and Dnmt3b. Deletion of the *Dnmt1* gene in the developing mouse CNS causes neonatal death because of respiratory failure. An enriched environment causes upregulation of Dnmt1 mRNA, indicating a role in learning and memory (for a review, see Feng *et al*., 2007). Furthermore, Rett syndrome, which is a neurodevelopmental disorder, is caused by mutations in a protein, MeCP2, which binds to methylated CpG dinucleotides (Amir *et al*., 1999), and mutations in *DNMT3B* result in the ICF (immunodeficiency, centromere instability, and facial anomaly) syndrome, and some ICF patients also have mental retardation (Xu *et al*., 1999).

Recapitulation of CNS development in stem cells cultured in vitro

Given the expanding knowledge in terms of molecular programs required for CNS development in vivo, it is becoming increasingly clear that this information can be used to begin to recapitulate aspects of neuronal differentiation in cultured cells. This is likely to become important for future transplantation therapies in neurodegenerative disease and after CNS injury. In particular, progress has been made with regard to differentiation of embryonic stem cells, which are pluripotent cells derived from the preimplantation embryo (Murry & Keller, 2008). Protocols have been established for directed differentiation of embryonic stem cells to many lineages and mature cell types, and this is also true for the CNS. Neural progenitors, capable of generating neurons, astrocytes, and oligodendrocytes, can be generated from embryonic stem cells, as well as induced pluripotent stem (iPS) cells, and reproducible differentiation of both motor neurons (Wichterle *et al*., 2002) and dopaminergic neurons (Andersson *et al*., 2006) has been reported. In both these cases, the successful protocols were based on recapitulating in the Petri dish, combinations of extrinsic factors and transcription factor programs that exist in the developing CNS in vivo.

Conclusions

Rapid progress is currently being made in deciphering the molecular programs that control the early aspects of CNS development. As discussed in this chapter, many of these molecular programs are evolutionarily highly conserved. The increased understanding of the developmental biology of neural tube closure, formation of layers in the cortex, and patterning along the rostrocaudal and dorsoventral body axes, as well as new insights into epigenetics, have also led to the identification of some of the genes involved in human CNS malformations.

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Historic box 1 The discovery of the neuronal organizer
Hugo Lagercrantz

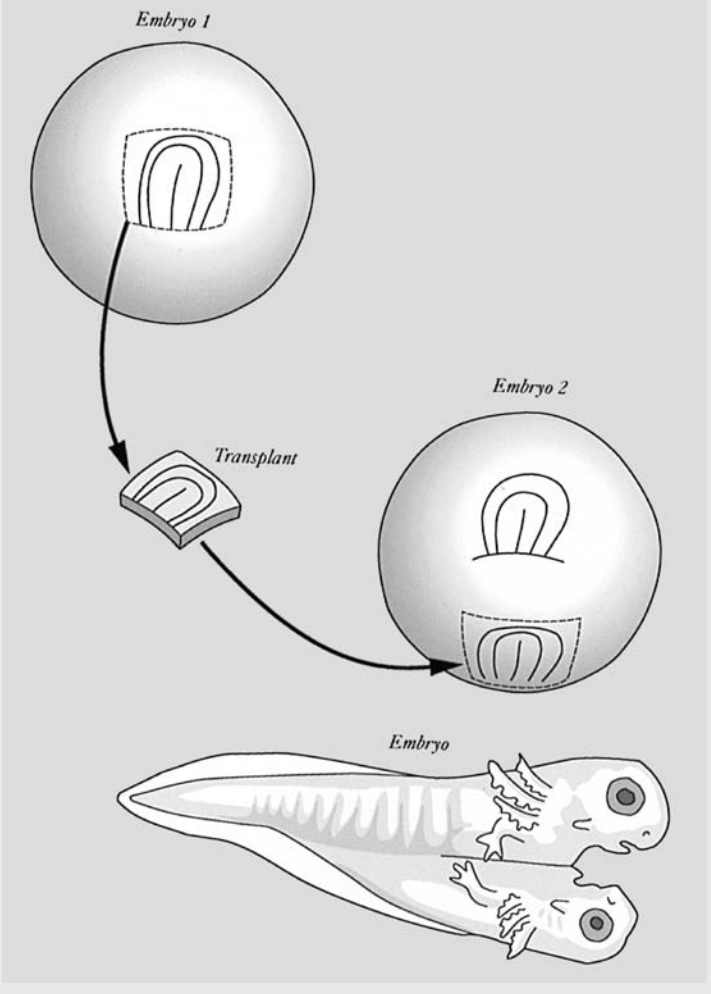

Fig. HB1 Mangold and Spemann demonstrated that, if a piece of a salamander embryo containing the neuronal organizer is transplanted to another embryo, a salamander with two heads can be formed. (Drawing by S. Söderlind.)

Spemann became interested in experimental embryology, and by developing micro-manipulative techniques and "cutting, pasting, and painting" he discovered the phenomenon of induction, i.e., the differentiation of cells in the embryo to become particular tissues is dependent on a stimulus from adjacent tissues.

To further explore this finding, Spemann suggested that one of his students, Hilde Mangold, should study induction phenomena in newt embryos. Mangold had studied arts and philosophy, but when she heard a lecture by Spemann, she was so impressed that she asked to work on a project. Spemann suggested that she could try to transplant the dorsal blastopore lip material into another embryo. This corresponds to the node on the epiblast, from where cells migrate to form the mesoderm. She then discovered that some of these embryos developed two heads, sometimes with only a rudimentary brain but also sometimes with two complete brains (Fig. HB1). The second head was formed mainly from cells of the host rather than the donor, indicating that the nervous tissue was induced by some factor.

Mangold submitted her thesis in February 1923 and was examined in philosophy by Edmund Husserl, who found her performance very satisfactory. One year later she died tragically. As a German wife she had to devote her life to *Kinder und Küche*. While warming food for her baby, she set herself alight after refueling a stove and died.

Hans Spemann received the Nobel Prize in physiology or medicine in 1935 mainly for this discovery of the organizer, later called Spemann's organizer. One of his most successful disciples, Viktor Hamburger, left Germany and started a new career in St Louis, Missouri, USA. He became mentor for Rita Levi-Montalcini (see Historic box 3).

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Chapter 3

Holoprosencephaly and microcephaly vera: perturbations of proliferation

Verne S. Caviness Jr., Pradeep G. Bhide, and Richard S. Nowakowski

Malformations of the developing nervous system may arise from disorders primarily affecting the nervous system or arise incidentally as part of multisystemic disorders. More than a century of study preceding the modern era of cellular and molecular biology has established a schema for the basic histogenetic sequences of development that are disrupted by the molecular and cellular biological perturbations which give rise to the most well-known developmental malformations of the brain (Sidman & Rakic, 1982; Caviness *et al.*, 2008). These encompass histogenetic processes ranging from cell proliferation to cellular events involved in elementary pattern formation including migration and postmigratory rearrangements, to the processes of growth and differentiation, including the winnowing of "excess" cells and cell processes. The perturbations arising from genetic mutations are among the most extreme in their consequences for development of the nervous system and for adaptation or even survival of the organism. They have, however, offered penetrating insights into the nature and mechanisms through which genetic regulation governs the cellular and molecular biological processes through which development proceeds.

Our focus over the years has been the proliferative process, the earliest step in the histogenetic sequence. In particular, we have focused on regulation of proliferative mechanisms that give rise to the vertebrate neocortex, with the mouse being our experimental model. Over time, this work has served to inform our parallel interest in disorders of the developing human forebrain as encountered in the clinic. This chapter focuses on the role of the proliferative process in the origin of two general classes of human cerebral malformation, each reflecting in different and informative ways on the fundamental regulatory mechanisms of neuron specification and proliferation. We will treat only the origin of projection neurons arising in the juxtaventricular epithelium of the neocortical embryonic brain. The γ-aminobutyric-acidergic (GABAergic) interneurons have a fundamentally different histogenetic history with their origin in basal forebrain proliferative structures (Wonders *et al.*, 2008). Whereas substantial evidence supports the postnatal origin of neurons in the dentate gyrus of the postnatal hippocampus (Kornack & Rakic, 1999; Breunig *et al.*, 2007), the status of postnatal origin of neocortical neurons has been debated and is beyond the scope of this discussion (Nowakowski, 2006). The spectrum of disorders collectively to be considered with respect to the origin of neocortical projection neurons will be grouped as holoprosencephaly (HPE) and the heritable spectrum of microcephaly vera (MV).

Synopsis of early central nervous system development

Before discussing the abnormalities that occur in development, we present here a brief synopsis of the normal events and their sequence. With respect to cell proliferation in the developing central nervous system (CNS) there are, in essence, three time periods:

- an initial period of expansion and regional differentiation;
- a neuronogenetic period;
- a gliogenic period.

The initial period of expansion and regional differentiation begins as the neural tube forms and lasts through approximately through the eighth week of gestation. During this period the major subdivisions of the CNS form along the long axis of the neural tube (Nowakowski & Hayes, 1999). Simultaneously with this, regional specializations develop tangential to the

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long axis of the neural tube. During this period, cell proliferation occurs and the size of the nervous system increases, but no neurons are produced so essentially all of the production is devoted to the expansion of the developing CNS.

The beginning of the neuronogenetic period is marked, quite logically, by the production of the first neurons. For the neocortex, this begins at about the eighth week of gestation and continues until about the twenty-fifth week of gestation, which corresponds to the end of the period of neuron production. During this time period the developing neocortex expands tremendously (Caviness *et al*., 1995; Nowakowski *et al*., 2002). In surface area alone the neocortex expands well over 100-fold, and it also increases dramatically in thickness as new neurons are produced. The two chief processes that occur during this period are cell proliferation, to produce the neurons of the cortex, and neuronal migration, to move the neurons from the site of production to their final position. Neuron production occurs in the ventricular zone, a narrow zone of proliferating cells that lines the lateral ventricles of the brain. It is the cell production in the ventricular zone that drives the tremendous expansion of the neocortex (Caviness *et al*., 1995; Nowakowski *et al*., 2002). The neuronal progenitors in the ventricular zone form a pseudostratified ventricular epithelium (PVE). Many of the PVE progeny are permanently postmitotic and migrate to the cortex following a terminal division within the ventricular zone. Those that do not migrate directly to the cortex instead undergo one or more amplifying divisions in the subventricular zone before migrating to the cortex (Noctor *et al*., 2007). Postmitotic neurons, whether arising terminally from the PVE or the subventricular zone, migrate to the neocortex itself in the outer part of the cerebral hemisphere by moving along the surface of radial glial cells. Both cell proliferation and the subsequent neuronal migration are highly regulated processes. Our focus here is primarily on the PVE and the ventricular zone.

The nuclei of PVE progenitors appear to be evenly distributed within the epithelium. This even distribution, however, hides a dynamic proliferative behavior during which the nuclei of the cells of the ventricular zone participate in a complex "dance" that is highly correlated with the phases of the cell cycle (Fig. 3.1(*a*)). The dance begins at the ventricular surface where the proliferating cells divide. Then the cells enter G1 and move into the outer half of the ventricular zone, where they enter the S phase to synthesize DNA. Next, the cells enter G2 and move rapidly back to the ventricular surface to divide again during M phase. This cell cycle-associated dance is estimated to occur ~35 times during the development of the human neocortex (Caviness *et al*., 1995; Nowakowski *et al*., 2002). With each pass through the cell cycle, each cell becomes two, but the daughter cells do not both reenter the cell cycle. Instead with each pass through the cell cycle, a steadily increasing proportion of the daughter cells exits or quits the cell cycle (Nowakowski *et al*., 2002). The exiting cells then migrate to the cortex using radial glial fibers as guides (Fig. 3.1(*b*)). This radial migration forms the basis of the radial unit perspective on neocortical development (Rakic, 2007). Each cell that exits the ventricular or the subventricular zone becomes apposed to a radial glial fiber and migrates from the proliferative zones to the top of the cortical plate. Disruption of migration will lead to ectopic neurons that may not get wired into the final cortex circuitry appropriately.

After the involution and disappearance of the ventricular zone around the twenty-fifth week of gestation cell proliferation continues for several more weeks in the subventricular zone. This is the period of gliogenesis, as only glial cells are produced during this time. Recent results indicate that few, if any, neurons are produced in the neocortex in the postnatal and adult periods (Bhardwaj *et al*., 2006; Nowakowski, 2006).

Holoprosencephaly

HPE is a term that groups together a wide range of malformations in which normally paired and separate forebrain structures are instead continuous across the midline (Kundrat, 1882; DeMyer, 1977; Cohen, 2001). HPE occurs in widely ranging vertebrate species and involves telencephalic and typically diencephalic structures. In primates, including humans, it also involves the neocortex. A traditional clinical-anatomical classification of the diverse forms is based on the degree of separation of the cerebra (Kundrat, 1882; DeMyer, 1977; Cohen, 2001). Traditionally it is accepted that there are no bilaterally paired forebrain structures in the *alobar* form of HPE. In the *semilobar* form of HPE, there is unpaired continuity rostrally in the telencephalon but with pairing caudally with respect to an interhemispheric fissure. Finally, there is also a *lobar* form of HPE in which pairing is almost complete with respect to a midline interhemispheric fissure.

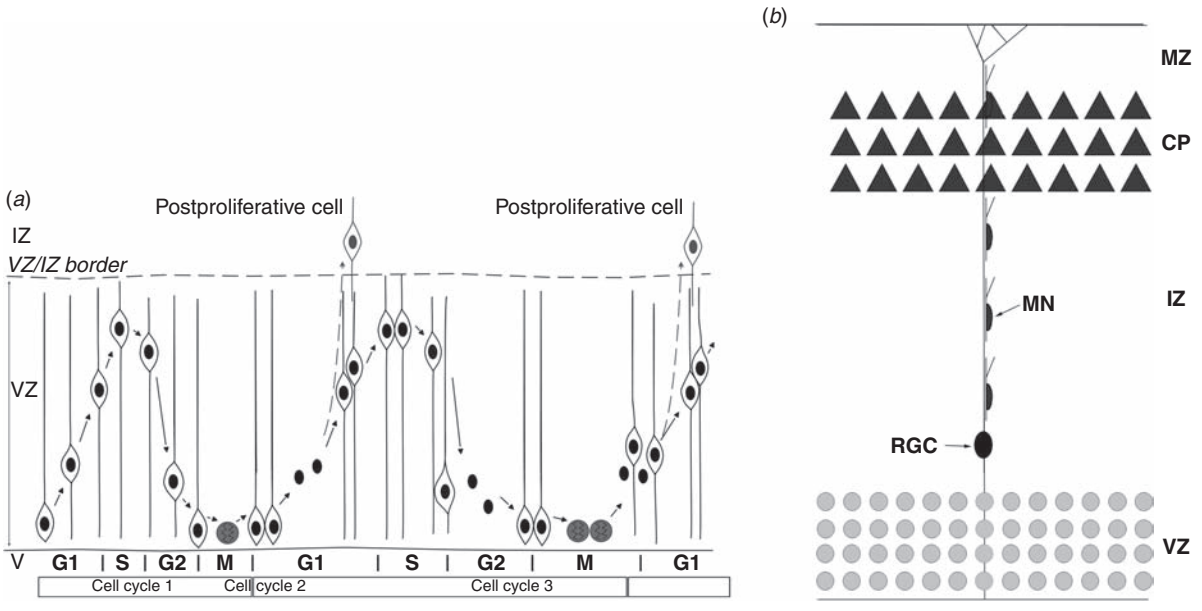

Fig. 3.1 (*a*) Within the ventricular zone the nuclei of the proliferative cells move "up and down" as they progress through the cell cycle. In humans, it is estimated that there are ~35 cell cycles during the time that the neocortex is being produced. At the completion of each cell cycle, some of the cells exit the cell cycle as postproliferative cells, which then migrate to the cortical plate. (*b*) The migration of the young neurons is guided by radial glial fibers that span the distance between the top of the proliferative zones and the top of the cortical plate. Each migrating neuron moves along the surface of a radial glial cell to reach its final position at the border between the cortical plate and the marginal zone. RGC, radial glial cell; MN, migrating neuron; VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate; MZ, marginal zone; G1, S, G2, and M, phases of the cell cycle.

This classification system is predictive of the degree of associated disability. Thus, the alobar form presents not only extreme neurological disability in an infant unlikely to survive the perinatal period, in extreme alobar forms there may also be craniofacial deformities, a proboscis, or an incompletely paired "cyclopic" eye (DeMyer & Zeman, 1963; Kobori *et al.*, 1987; Cohen, 1989). The malformation is common with an estimated minimum incidence at conception of 1/250 (Matsunaga & Shiota, 1977) but survival to term is < 1/20 000 (Bullen *et al.*, 2001).

HPE presents a disruption of mechanisms set in motion by morphogens diffusing from tissue adjacent to or from centers within the CNS that establish within the nascent CNS the coordinates of subsequent developmental transformations. We review here findings from seven brains, which illustrate the semilobar form of the malformation (Takahashi *et al.*, 2003, 2004). This form of HPE, limited to the telencephalon, illustrates inductive disruption expressed in the course of evagination of the cerebral hemispheres.

The forebrain is partitioned for present purposes into the distribution of three topological domains: a fully diencephalic (D) topological segment, the temporal limb of the interface of diencephalic and telencephalic junction (DT), and a fully telencephalic (T) topological segment (Takahashi *et al.*, 2003, 2004). Topological anomaly in the examples of the semilobar form reviewed here is confined to the T domain although in more extreme forms it may involve both the T and D domains. We have encountered no examples in our own series or in the literature where the DT domain is involved.

The telencephalic anomaly in the seven brains is notably uniform. First, there is rostromedial continuity across the midline of the normally paired and separate orbitofrontal and polar cortices, without an intervening interhemispheric fissure. Subcortically, there is continuity across the midline of the normally paired claustrum, caudate, and accumbens nuclei (Fig. 3.2), and variably the suprachiasmatic anterior hypothalamus (Takahashi *et al.*, 2003). A critical point is that, in all, there is complete absence of all septal structures and the septal limbs of the fornix, and choroid plexus. That is, structures that normally intervene between hemispheres, including those that seal the foramen of Monro, are frankly absent. As a

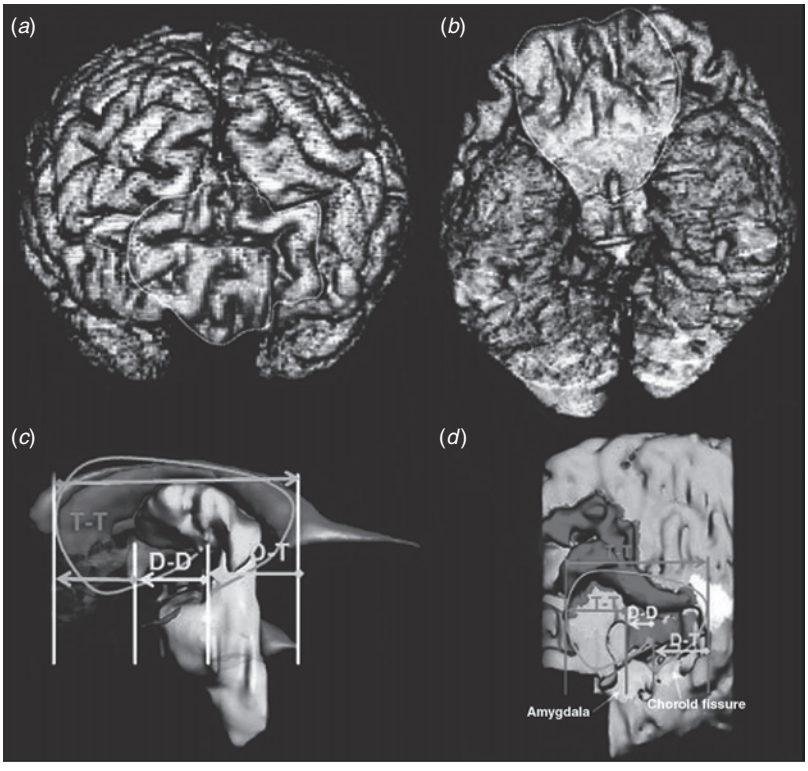

Fig. 3.2 (See color plate section.) Anatomic overview of holoprosencephaly (HPE). (*a*) Frontal and (b) inferior views. A veil is superposed over the rostral conjunction of the left and right cortices with no intervening interhemispheric fissure, which emerges immediately caudal to the veil. (*c*) Topological segments: D-D, the diencephalic segment extends from the subthalamus to the suprachiasmatic junction with the telencephalon. T-T, the telencephalic segment, extends from the D-D segment through the septal limb of hemisphere to the hippocampal commissure. D-T, the diencephalic-telencephalic segment, diverges laterally from the midline to follow the temporal limb of the choroid fissure to the amygdala. (*d*) The seam and adjacent structures as seen from the midsagittal aspect in HPE (Takahashi *et al.*, 2003). (With permission of Oxford University Press.)

consequence, the septal limb of the choroidal fissure is splayed open with continuity of the bihemispheric and diencephalic ventricular volumes, allowing ready recognition of the HPE malformation.

Second and variably, a midsagittal fissure emerges posteriorly and continues to deepen with posterior progression. The fundus of the fissure forms the midline of the ventricular roof. The fundus is continuous with corresponding infolded gray matter that is continuous with the cortex of the opposite hemisphere. This fundic gray matter is part of a rostrocaudally continuous seam of gray matter extending forward along the roof of the ventricle, conjoining striatal masses anteriorly. It continues caudally beyond its continuity with the convexity and interhemispheric cortex at the surface of the hemisphere to end where it abuts a rudimentary corpus callosum. Thus, despite the absence of the normally intervening dorsomedial structures in the midline, the gray matter masses of the two hemispheres are still joined by a bridging gray seam. They are not fully separated by a cleft as might have been expected had the absent structures been destroyed by a late-acting secondary pathological process, as is the case with porencephaly (Levine *et al.*, 1974). Whereas the developmental history of the seam cannot be unraveled from the available human material, such a structure is evident in a mutant murine form (see Rash & Grove, 2007). In its signal intensity characteristics, form, and location it resembles periventricular heterotopia (Fox & Walsh, 1999; Sheen *et al.*, 2002); that is, cells of the proliferative epithelium that underwent their terminal division without acquiring any of the specialized features of the differentiated laminar classes of the cerebral cortex.

Third, within the T domain the brains are disproportionately small. Estimates for the average age-adjusted volumetric deviation for the total brain for the HPE series with wide variation was about 55% (52%, 51%, and 68% for forebrain, telencephalon, and diencephalon, respectively). In each brain, the T segment component with full depth fissure and seam was relatively longer than the anterior segment with no fissure or the intermediate zone with no or incomplete fissure (Fig. 3.2). That is the degree of volumetric reduction correlated with the degree of topological abnormality.

The formal origin of the semilobar form of HPE has been classically traced to the induction of telencephalic hemisphere evagination following

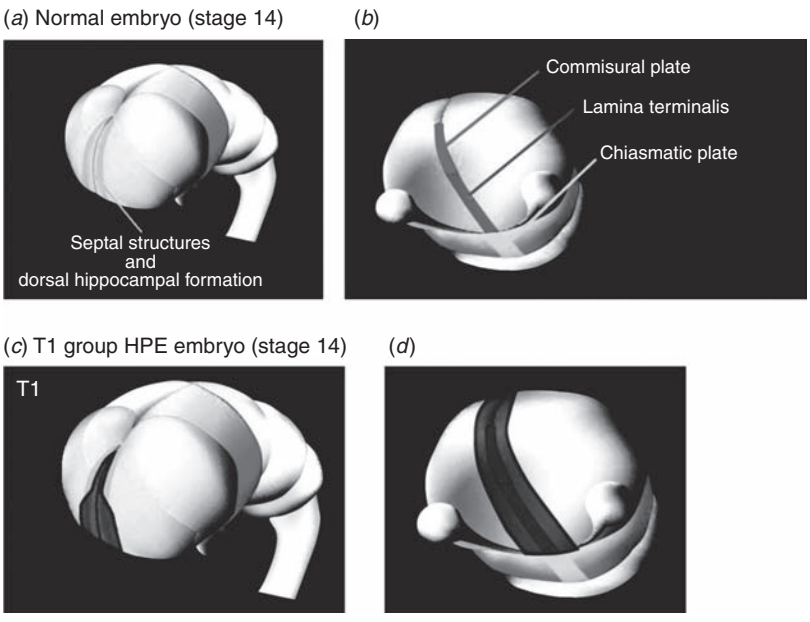

Fig. 3.3 Schematic representations of a stage 14 human embryo. (*a*, *b*) The cerebral hemispheres emerge as bilaterally paired evaginations from the single telencephalic vesicle of the terminal neural tube (Muller & O'Rahilly, 1988). The anterior arcs of evagination of the right and left vesicles are paramedian in position and run parallel within the telencephalic vesicle. The posterior arcs of evagination of the right and left vesicles, by contrast, diverge laterally from the midline to follow the right and left telencephalic–diencephalic borders. That is, the telencephalic rims of the two hemispheres arise from the midline but the telencephalic–diencephalic rims of the two hemispheres emerge from opposite sides of the neural tube (*c*, *d*) (Takahashi *et al.*, 2003). (With permission of Oxford University Press.)

neurulation. Normally each hemisphere evaginates along a continuous margin that extends caudally from the ventrorostral suprachiasmatic diencephalic–telencephalic junction, through the midline rostral telencephalic anlagen, where the two hemispheres are separated by bilateral septal and cortical hem anlagen (Fig. 3.3). Caudal to the level of the topological abnormality, the margins of the two hemispheres diverge normally along the dorsal diencephalic–telencephalic boundary to follow a laterally and ventrally coursing path toward the basal medial tip of the temporal lobe. We infer from the present observations that there must also be downregulation of neuron production generally but with a regional emphasis on the primarily affected topological domain.

Microcephaly vera

Microcephaly literally means small brain, but traditionally this term has been used where the measured head circumference is less than two, or, for some, three standard deviations below the mean (Mochida & Walsh, 2001; Woods, 2004). It may be a consequence of virtually any grave disorder affecting the brain during its prenatal and postnatal growth cycles. For present purposes, the term is applied to microcephaly vera, a spectrum of microcephaly caused by autosomal recessive mutation and expressed morphologically prior to the thirty-second gestational week (Mochida & Walsh, 2001; Woods, 2004). Only the brain develops abnormally, with brain weights in some instances in the region of 400 g, corresponding to that of the brain of an adult chimpanzee (Richards, 2006). Classical studies of the physiognomy and the neocortical cytoarchitecture associated with vera were published late in the nineteenth century (Cunningham, 1895; Hammarberg, 1895; Caviness *et al.*, 2008). Primary and even secondary gyri of the neocortex are recognizable but higher order surface folds typically are not. Architectonic abnormalities are limited to the neocortex where there is major impoverishment of the neuronal populations of the supragranular layer but a relative preservation of the cells of the infragranular layer (Hammarberg, 1895; Caviness *et al.*, 2008) (Fig. 3.4). Importantly, all laminar classes appear from general cell stains to have been formed (and therefore specified) and all appear to be in appropriate laminar order, in that there is no heterotopia along the subcortical and intracortical migratory routes and tangential ordering of lamina is regular. That is, all histogenetic processes leading to the normal topology of pattern formation appear to have been executed without flaw. However, the architectonic evidence implies that it is the late-formed neurons, those assigned to superficial layers, which are preferentially impoverished (Hammarberg, 1895; Mochida & Walsh, 2001; Caviness *et al.*, 2008).

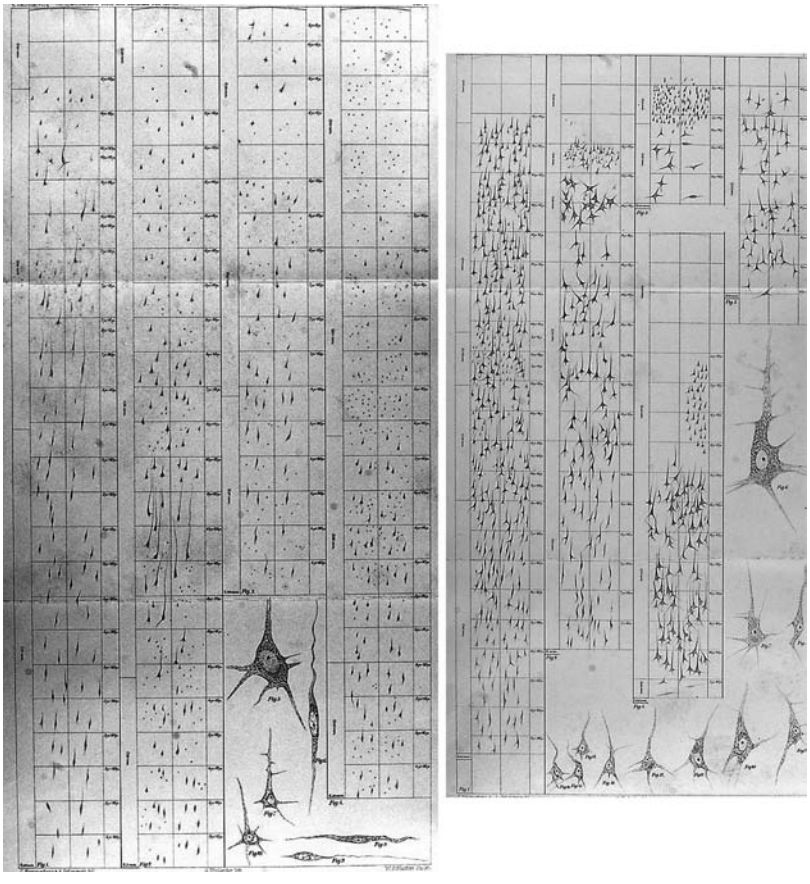

Fig. 3.4 Laminar patterns in microcephaly vera. Architectonic patterns of normal cortex (left panel) and cortex from microcephalic subjects (right panel). The Betz cells in column 3 and 4 of the left and right panels identify the pre-Rolandic gyrus in normal and microcephalic brains. The other columns in each panel illustrate corresponding regions of frontal, parietal, and occipital association cortical regions. The microcephalic cortex is laminated with attenuation of superficial layers. (From Hammarberg, 1895; Caviness *et al.*, 2008, with permission from S. Karger AG, Basel.)

Molecular genetic and morphological analyses of HPE and microcephaly vera

Typical and extreme forms of HPE have arisen from environmental exposures, specifically fetal exposure to the plant alkaloid cyclopamine in ungulates or ethanol in chicks, and the incidence is somewhat heightened in association with diabetes in humans. However, the principal determinants of the malformation appear to be genetic mistakes (Cohen & Shiota, 2002). Thus, at least six discrete genetic loci (six3, TDGF1, Shh, Ptch, Zic2, TGIF) and as many as 12 chromosomal anomalies have been implicated in the origin of this malformation in the human condition and more than a dozen other vertebrates, in particular mouse and chick (Cohen & Shiota, 2002; Roessler & Muenke, 2003; Roessler *et al.*, 2003). Those thus far associated with the malformation in humans account for less than 20% of the probands that have undergone molecular genetic or karyotype analysis (Muenke & Cohen, 2000; Cohen & Shiota, 2002). However, center stage has been the gene for the diffusible ligand sonic hedgehog (*SHH*) and its transmembrane receptor patched (*PTCH*), elements of the hedgehog signaling pathway. This signaling system has not only been implicated in regional and cell class specification along the entire length of the neural axis including the forebrain, but it has also been found to have mitogenic effects in widely varying systems (Edlund & Jessell, 1999; Solecki *et al.*, 2001).

A series of four genes has thus far been implicated in the origin of microcephaly vera. Genetic analysis of candidate mutations giving rise to the microcephaly vera spectrum of disorders has mapped to no fewer than six linkage groups referred to as MCPH1–6 (Mochida & Walsh, 2001; Leal *et al.*, 2003; Woods *et al.*, 2006). Of these loci only for two has the gene been identified. These are MCPH1, or microcephalin, and MCPH5 or ASPM (abnormal spindlelike microcephaly). All impact on the proliferative operations of cycling cells with three of the four encoding proteins thus far identified as mutated gene products integral to

the structure or operation of the mitotic spindle. Because of the effect of these genes on brain size they have been suggested to figure in evolutionary mechanisms of trans-specific advances in brain size and complexity of behavior (Richards, 2006). However, variations in these genes within human populations have not correlated with either variations in brain size or IQ (Kouprina *et al*., 2005; Woods *et al*., 2006).

HPE and microcephaly vera: disorders primarily of proliferative mechanisms

The proliferative epithelium of the telencephalon expands from the proliferative epithelium of the neural tube which, in turn, expands from that of the neural plate. This epithelium generates almost all neuronal and glial elements of the CNS. The mechanisms that establish regional distinctions and specify the diverse neuronal classes of the CNS are also set in motion in the course of the proliferative process as it operates in the epithelium (Rakic, 1988; Miyashita-Lin *et al*., 1999; Grove & Fukuchi-Shimogori, 2003). HPE appears to represent genetically determined disruption of mechanisms of both cell production and specification of projection neuron laminar class. Thus, the brain is small without evidence of a secondary destructive process. The cell populations of the dorsomedial midline are frankly absent. To the extent that the seam represents unspecified heteropic cells, its mass is certainly small relative to the expected expansion of the founder population for this group of cells in the normal brain. Presumably these populations were never specified. We, therefore, consider that the seam represents a population of cells where the critical attributes of cell class including those necessary to migration and pattern formation were not specified. This was associated with a marked reduction in output from the unspecified population.

The morphologic analyses of microcephaly vera also direct attention to the primary mechanisms that regulate cell production, but cytoarchitectonic analysis indicates that production is here dissociated from specification. In contrast to HPE where the cell types native to an entire topological domain are variably absent, architectonic studies have found all the principal cell types of the neocortex to be present in microcephaly vera but with a reduction in the cell number of the superficial cortical layers. As with HPE there is no indication of an intercurrent destructive process. In that neuronal origin follows a deep to superficial layer sequence, this implies that the process is increasingly expressed as the proliferative process advances. Moreover, in contrast to semilobar HPE, where rostromedial telencephalon is affected, the anomaly in microcephaly vera is expressed throughout the dorsal telencephalon.

HPE and microcephaly vera: integration of specification and neuron production

The inferences from anatomy and cytology respecting HPE and microcephaly vera direct attention to the proliferative epithelium of the neocortex as an organ that coordinates regulation of neuron production and specification. Whereas regulation of proliferation has been studied in most detail in rodents, these mechanisms appear to be identically regulated across all mammalian species. Thus, the proportion of the neurogenetic interval given to the origin of each successive laminar class is uniformly scaled across all mammalian species extending from rodent to primate (Caviness *et al*., 1995). Moreover the properties and topology of arrangement of diverse cortical representations are essentially identical across the full mammalian spectrum from protherians to eutherians (Krubitzer & Kaas, 2005). We consider in the discussion to follow, a model whereby a critical mechanism of the coordination of neuron production and specification is an opponent interaction of the Notch signal transduction mechanism and the cell cycle inhibitor $p27^{Kip1}$.

The proliferative epithelium

Our model for the principal regulatory events of proliferation giving rise to neocortical projection neurons is based on studies in mouse over the interval E11–14. During this interval, the tangential extent of the epithelium is achieved and virtually all neuronal classes included in layers VI–II have been specified. Secondary expansions of the population occurring in the subventricular zone have not been shown to include further advance in the specification sequence (Noctor *et al*., 2004). The proliferative epithelium is a pseudostratified epithelium and because it is only one component of the ventricular zone, we have referred to it as the PVE. As with other histogenetic pseudostratified generative epithelia, nuclei of cells are distributed through its depths according to their proliferative phase, and the proliferative process is asynchronous.

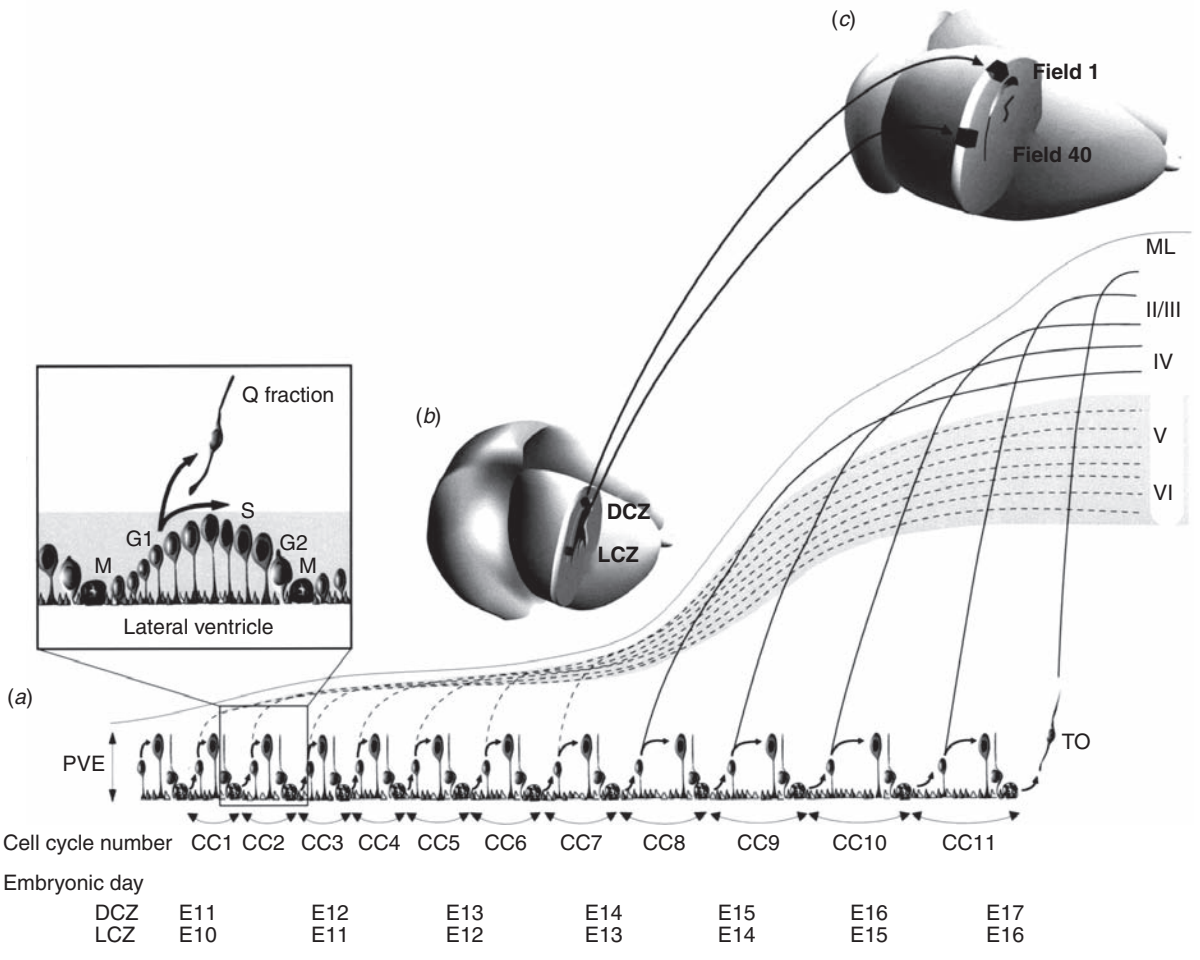

Fig. 3.5 Neurogenesis in the murine pseudostratified ventricular epithelium (PVE). (*a*) The founder proliferative populations of dorsomedial and lateral cortical zones (DCZ and LCZ, respectively) execute 11 cell cycles (CC1–CC11) over the course of the six-day neurogenetic interval, E10/11 to E16/17. The cycle sequence is advanced by ~24 hours in LCZ. Interkinetic nuclear migration is shown for CC2 in the inset image. Neurons leaving the VZ of the embryo (*b*) with successive cycles are destined systematically with each cycle to successive deep to superficial layers of the adult brain (c). (From Takahashi *et al.*, 1999, with permission.)

We emphasize here four features of the neocortex that bear upon the proliferative output of the epithelium. First, the neocortex is a six-layered structure, in which projection neurons of a common class dominate their respective layers II–VI through the entire neocortex. Variations in the cytological features, and the specific patterns of arrangement of each class from region to region, are characteristic of each of the diverse architectonic representations of the neocortex (Caviness, 1975). That is, in the radial axis of the cortex there is a laminar succession ordered by general neuronal class. In the tangential axes, there are variations in the specific laminar features according to the neocortical architectonic representations. These regional differences notwithstanding, the neurons of each of the laminar classes throughout the neocortex arise in a systematic overlapping succession with the earliest cells being destined for the deepest layers while progressively later formed cells are destined for progressively more superficial layers (Fig. 3.5) (Angevine & Sidman, 1961; Bisconte & Marty, 1975; Caviness, 1982; Bayer & Altman, 1991).

Secondly, the lineages of the epithelium are equipotent, each lineage on average giving rise to the same canonical sequence of laminar projection neuronal classes over a succession of cell cycles (Walsh & Cepko, 1990). That is, on average each lineage gives rise to the full range of neuronal classes with no lineage

specialized for the separate cell classes (Walsh & Cepko, 1990; Cai *et al*., 2002). Although the sequence is systematic, there is marked overlap such that at any moment through much of the neurogenetic interval spatially adjacent lineages will be giving rise to neurons destined for two or even three different layers.

Third, the sequence unfolds according to a transverse neurogenetic gradient (TNG) (Bayer & Altman, 1991). The sequence begins at the rostrolateral extreme of the neocortex, i.e., the head of the TNG, and progresses down a gradient, i.e., dorsomedially. This requires that each lineage at all times in the neurogenetic interval must know "where" it is with reference to the full expanse of the epithelium. It must know "when" it is with respect to its order of the laminar class specification sequence.

Parameters of proliferation

The proliferative and specification mechanisms are closely coordinated. The proliferative process of the PVE, just as the neurogenetic succession, advances as a gradient aligned with the TNG. Over the course of the neurogenetic interval in the mouse, the duration of the cell cycle (T_C) in the PVE increases stepwise with successive cycles from ~ 8 to ~20 hours with increases in T_C due exclusively to increases in the duration of the G1 phase of the cycle (T_{G1}). The durations of other phases of the cycle are not regulated. Concurrently there is a cycle-by-cycle increase in the fractional output of the cycle (Q; the complementary fraction that reenters the S phase is designated P; Q + P = 1.0) (Takahashi *et al*., 1995; Miyama *et al*., 1997; Caviness *et al*., 2000). Overall, each proliferative lineage will execute 11 integer cell cycles over its neurogenetic interval. We add that the slope of the TNG is a function of T_C while the total number of cycles in the interval is governed by the fractional advance of Q with each cycle (Caviness *et al*., 2000).

A proliferative model: consequences of perturbation

It follows from this model that the number of cells to arise in the neurogenic interval will depend on the size of the founder population and will be a function of cycle number and Q per cycle. Perturbations that advance the cycle-by-cycle increase in Q would prematurely deplete the proliferative pool of the PVE and secondarily also impoverish the subventricular zone progenitors. Collectively, it would lead to a substantial impoverishment of neurons, which would be felt disproportionately in progressively later formed, more superficial layers. As a model it has been validated in mouse with perturbations that increase Q, including $p27^{Kip1}$ overexpression and Lmx2, id2, and tlx knockout *inter alia* (Monuki *et al*., 2001; Roy *et al*., 2004; Yun *et al*., 2004; Tarui *et al*., 2005). Such a mechanism plausibly accounts for the pattern of neuron impoverishment of the superficial layers in microcephaly vera. A model for HPE must be more complex in that the reduced numbers of neurons is associated with a failure of specification of dorsal midline cell classes.

Specification: gradient models

The mechanisms underlying specification relative to those of proliferation have been more illusive. To return to a theme introduced earlier, each proliferative lineage must "know" where it is in the TNG and "when" it is in relation to the succession order of the cells to which it is giving rise. It has been traditionally held that this information is conferred by mechanisms inherent in a gradient of a diffusible morphogen aligned with the principal axis of the epithelium. Specification and origin of successive neuronal classes are a consequence of differential transcription induction at critical threshold concentrations along the gradient (Jessell & Melton, 1992; Wolpert *et al*., 1998; Gurdon & Bourillot, 2001; Grove & Fukuchi-Shimogori, 2003). Inductive gradients of a diffusible morphogen, arising initially from sources external to the CNS anlage but ultimately from secondary sources within the CNS, satisfy the principal specification requirements of the neural tube (Edlund & Jessell, 1999). Telencephalic development appears to go forward within a cartesian coordinate system where fibroblast growth factor (FGF)-3, -8, -17, -18, Shh, and retinoids act at the anterior pole, and Wnts and Bmps at the medial–lateral axis (Rash & Grove, 2007). That is, the axis of alignment of these coordinates from rostrolaterally to caudomedially more or less is that of the TNG (Grove & Fukuchi-Shimogori, 2003). In the cerebrum, however, no morphogen gradient has been found to be established either by diffusion or some other indirect means even across the short range of action that would be required by the smallest mammals, much less across the vast expanse that would be necessary in primates and other large mammals (Grove & Fukuchi-Shimogori, 2003).

The above considerations have led to a shift in attention from the search for extrinsically arising diffusible morphogens to epithelium-intrinsic transcription

factors implicated by gain and loss of function analyses to be essential to regional and cell class specification within the cortex. A large and increasing number of these have been found to be distributed throughout the PVE, where these are expressed according to gradients aligned with the principal axis of the epithelium (Puelles & Rubenstein, 1993; Cai *et al*., 2000; Monuki *et al*., 2001; Schuurmans & Guillemot, 2002; Grove & Fukuchi-Shimogori, 2003). If the homeodomain transcription factor Lhx2 is taken as representative, however, this model also fails in a detailed analysis of its gradient.

Thus, the expression gradient of Lhx2 may satisfy the "where" requirement but it does not satisfy the "when" requirement. However, the intersection of gradients of Lhx2 expression and that of T_{G1} uniquely defines both the "where" and the "when" of a proliferative lineage and in principle in this sense satisfies the requirements of a model of specification. Moreover this transcription factor–proliferative parameter model is validated in the sense that overexpression of $p27^{Kip1}$ both advances the gradient of Lhx2 and leads to an early exit of neurons from the proliferative pool. Evidently overexpression of $p27^{Kip1}$, as predicted by the model, leads the proliferative lineage to think it is operating at a more advanced T_{G1} and is engaged in a more advanced cell cycle than it actually is (Suter *et al*., 2007).

Architecture of the cell cycle

The implications of anatomical analysis of HPE and microcephaly vera, taken together with the proposed model to coordinate neuron production and cell specification, direct attention to the architecture of the eukaryotic cell cycle. The mechanisms of this cycle are generalized to developmental systems and are greatly robust, reflecting redundancy with complex interleaving of interacting molecular mechanisms variably sensitive to signals and with nonlinear throughput kinetics. In yeast there are as many as 800 genes involved in the regulation of the cell cycle (Ubersax & Ferrell, 2006) whereas in vertebrates, and especially in mammals, there are as many as 100 regulating the G1 to S switch alone (Pardee, 2004), with the total acting upon the cycle considerably greater (Kohn, 1999). This complexity notwithstanding we are served for present purposes by the following general features. First of all the eukaryotic cell cycle is a bistable system where the proliferating cell toggles between stable G1 and the stable S-G2-M phases (Ferrell, 2002; Novak & Tyson, 2004). Second, in these repetitive phase shifts, operations are replete with positive and negative feedback loops, including autocatalytic mechanisms. Third, product throughput at critical interactions surges according to nonlinear kinetics after time delays appropriate to cyclic behavior of the entire system.

The molecular switches that toggle between states are the cyclins, regulatory cofactors for a spectrum of kinases that phosphorylate and activate the principal molecular actors that implement the cycle. Among these are Cdk4/6 (cell division kinases) with cyclin D and Cdk2 with cyclin E, critical to the G1 to S transition, and Cdk1 with cyclin B, critical to the M to G1 transition. As requisite to the operation of the process there are both positive and negative feedback mechanisms at every node of protein interaction in this system. All have nonlinear reaction characteristics that may result in orders of magnitude accelerations in product throughput (Ferrell, 2002). The critical positive regulatory operation driving phase transitions is transcriptional upregulation of the cyclins whereas that stabilizing after a phase shift is ubiquitination and proteolysis of the cyclins (Sutterluty *et al*., 1999; Ganoth *et al*., 2001; Ang & Harper, 2004; Novak & Tyson, 2004). In steady-state systems on the other hand, transcription rates of the kinases and other critical components including cycle inhibitors and transcriptional activators and repressors are relatively constant with their shifts in functional state depending principally upon posttranslational modifications, especially phosphorylation and dephosphorylation.

A lynchpin of progression of the cell cycle is the transcriptional repressor Rb 105 (Novak & Tyson, 2004). In its unphosphorylated state Rb is a repressor of the transcription factor E2f. The phosphorylation of Rb detaches it from E2f, unleashing a cascade of transcriptions of the principal molecular operators required for the G1 to S transition including not only cyclin E/A but also RNA polymerases and a battery of housekeeping genes.

Go–no-go and specification

HPE and microcephaly vera both reflect an imbalance in mechanisms of cell production favoring premature cell cycle exit. HPE additionally reflects a failure of specification of cell classes normally populating the ventrorostral midline of the telencephalon. Plausible lynchpins in mechanisms relating to these two processes are those related to regulation of the signal transduction agent, notch1, driving the proliferative process and a regulator of specification and the cell

cycle inhibitor $p27^{Kip1}$ acting to drive cells out of a cycle.

Notch 1

The notch1 (of a family of four proteins) signal transduction system is a principal drive to proliferation in the neocortical PVE (Yoon & Gaiano, 2005). It is a bifunctional system in that it independently acts to restrain cell specification and differentiation. Notch is a single-pass transmembrane protein. It stands at the head of a complex battery of signaling mechanisms involving more than 24 components. The pathway is interactive with multiple other facilitatory or inhibitory systems with respect to proliferation and specification including the BMP-Wnt signaling mechanisms.

The notch receptor is activated by binding with jagged or delta ligands located on the surfaces of adjacent cells. Binding leads to cleavage of the notch intracellular domain (NICD), which translocates to the nucleus. In the nucleus it binds to the CBF1 repressor complex converting it to an activator of transcription. Among its multiple effects this complex upregulates bHLH transcription factors, preeminently the Hes and Herp (Hes-related proteins) family. These transcription factors are known to antagonize the transcription of proneural genes including Mash1 and the neurogenins.

In accord with these actions of this transduction system, the NICD protein is concentrated within the nuclei of cells in S phase expressing Hes1, but not in cells in M phase. Its distribution is complementary to proneural Mash1 in the ventral telencephalon and Ngn2 in the dorsal telencephalon. That is, it is not found in the postmitotic differentiating cells of the Q fraction. That is, it must be through some mechanism that involves downregulation of NICD that advance of the transcriptional program of the proneural genes is regulated. Thus, it is through cascades of transcription driven by the proneural genes that there is specification of differential neuron classes. There is great diversity in the architectonic and systems organization across the regions of the neocortex. It follows that there must be regional variations in the combinatorial levels of large arrays that give rise to the laminar classes that endow the specific properties of the diverse architectonic representations.

$P27^{Kip1}$

This cell cycle inhibitor, at the opposite pole from notch of the go–no-go proliferative tension, acts to block the entry of the G1 phase cell into S phase. It does so by holding cyclin E/Cdk2 levels below their activation threshold. Consistent with this action, the $p27^{Kip1}$ null state is associated with increase in brain and somatic organ size which is uniformly scaled suggesting that its mechanisms of action and weighting in the control of cell proliferation are relatively general and uniform (Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). Although the inhibitor has an independent effect on the mechanisms governing cell exit from the cycle, it is redundant with other agents in that these animals are limited in their sizes. Whereas it has been implicated in the cell biological functions necessary beyond cell cycle exit, including cell migration, it appears not to have a primary role in cell specification in the mammalian telencephalon. Thus cells of oligodendrocyte lineage derived from $p27^{Kip1}$ null mice undergo increased numbers of cell division under conditions of arrest but eventually do arrest despite the null state of $p27^{Kip1}$. It is of note that when proliferation does eventually arrest, they differentiate normally into oligodendrocytes (Casaccia-Bonnefil *et al.*, 1997, 1999).

Notch–$p27^{Kip1}$ opposition

Current evidence bearing upon regulatory interactions between notch and $p27^{Kip1}$ has emphasized a notch to $p27^{Kip1}$ control mechanism but not one operating in the reverse direction. This picture is certainly incomplete, but as it stands it carries our understanding of the proliferative mechanisms a few steps further. Obviously the effect of the notch system, acting to favor cell proliferation, is antagonistic to that of $p27^{Kip1}$ acting to restrain proliferation. Notch is directly antagonistic to $p27^{Kip1}$ and downregulates the inhibitor in two direct ways. That is, in this interaction we find a trace that directly integrates mechanisms regulating cell production and those of cell specification. The first of these mechanisms is direct transcriptional suppression of the $p27^{Kip1}$ protein. This is effected through direct Hes1 binding to consensus class C sites in 5′ flanking enhancer regions of the gene for $p27^{Kip1}$ (Murata *et al.*, 2005). The second mechanism is via ubiquitination and proteolysis. This is driven by Notch activation of transcription of Skp2 transcription via a CBF1-dependent mechanism (Sarmento *et al.*, 2005).

Where we are now and beyond

The discussion thus far has developed models for a series of complex cell biological mechanisms that

contribute to proliferation in the neocortical PVE. These models bear upon the mechanisms of cell production and specification. In using the general term "specification," we include both the radial mechanisms by which the broad laminar classes of the neocortex arise and those more specific mechanisms that underlie the origin of the regional differences tangential to the surface of the ventricle. In microcephaly vera where there is a general impoverishment of progressively more superficial layers without regional bias, it is the radial mechanism that has failed chiefly within the neocortical PVE. In HPE where there is a general reduction of telencephalic size, both the tangential and radial specification mechanisms have apparently failed. However, with HPE there is also a midline region in which all cells of specific regions are missing. We assume that this represents an absolute failure through a region-restricted mechanism of all mechanisms underlying the processes of specification.

We offer these models as grist for ongoing experimental design but fall far short of laying critical questions to rest. First, it is a model with general scope directed only at the radial processes of coordinated cell production and specification and does not approach those relating to regional, i.e., tangential, modulation of the specification process. Second, with respect to proliferative mechanisms, we point out that the model developed for notch–$p27^{Kip1}$ interaction is based on the assumption of steady-state operation of the proliferative process. That is, it is based on the assumption that the size of the proliferative pool remains constant with the number of cells exiting the cycle equal to that reentering it. It is under these conditions that the transcriptional rate of noncyclin operators is constant with toggle control vested principally in transcription and synthesis followed by proteolytic destruction of cyclins. In reality, the operation of the neocortical PVE is not steady state. Instead there is a cycle-by-cycle advance in Q from 0, prior to onset of the neurogenetic interval to 1.0 with a terminal round of cell division and exhaustion of the proliferative potential of the PVE. T_{G1} in coordinate fashion ascends cycle by cycle in an equally precisely regulated fashion, essentially quadrupling in duration over the course of the neurogenetic interval.

The dynamic progression of the neurogenetic process is associated with dynamic alterations in patterns of expression of essential operators. Thus as T_{G1} and Q increase with each cycle between E11 and E14, the transcriptional range of cyclin E falls whereas that of $p27^{Kip1}$ increases exponentially to low and high asymptotes, respectively (Delalle *et al.*, 1999). That is, these agents critical to the operation of the toggle between G1 and S reach their expression asymptote at a point in histogenesis where T_{G1} approaches its maximum and where Q = P = .5. At this point all cell classes are now expressed (and therefore specified). At this point, the PVE has reached its maximum tangential extent, that is the topology of the PVE protomap may be considered to be complete (Caviness *et al.*, 2000).

Where should one look for the overriding control mechanisms that govern this graded and greatly precise progression of cell production and specification? Is this through a graded regulation of the levels of notch or of $p27^{Kip1}$ or of other gradient control mechanisms that coordinately regulate both? For the present we can offer only fragmentary evidence that will serve for future experimental design. The first relates to the regulation of notch expression. Thus the levels of expression of the receptor are regulated by levels of expression of its surface ligands delta and jagged. In that these drive notch only from proliferating cells, it may be expected that the intensity of this drive will decrease as Q increases and a larger fraction of cells leave the cycle with each round of division (Ross *et al.*, 2003). A second fragment relates to the regulation of $p27^{Kip1}$ activity. Retinoids, highly diffusible through the extracellular compartment, are powerful upregulators of $p27^{Kip1}$ (Borriello *et al.*, 2006), a phenomenon associated with cycle arrest and cell differentiation. The mechanism is nuclear sequestration due to phosphorylation on the S10 residue of $p27^{Kip1}$. It is independent of effect on ubiquitination or transcription. The kinase responsible has not been specifically identified (Borriello *et al.*, 2006). With regard to this mechanism that might advance the neocortical neurogenetic proliferative process, little is known about the distribution of retinoids in the developing telencephalon other than exposure to exogenous sources is catastrophically teratogenic. It is of note, however, that in experimental systems that have examined this phenomenon, there is a dissociation of differentiation and proliferative regulation. Thus, an effect of retinoids to advance differentiation of exiting cells follows in intervals corresponding to only a fraction of the cell cycle length, while those altering cycle exit rate require one to two cycles. There is the additional point of interest that a latency of advance

in rate of cycle exit of one to two cell cycles is consistent with the finding that advance in Q is delayed for at least 24–48 hours in response to overexpression of $p27^{Kip1}$ (Tarui *et al.*, 2005). For whatever they are worth, these are leads that may point in the direction at least to mechanisms of histogenetic regulation based on a balance of antagonistic operations. These have never been approached before experimentally in the neocortex, the most complex of biological systems.

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Historic box 2 The neuron doctrine and proliferation of new neurons before and after birth
Hugo Lagercrantz

Although the cell theory was established in the middle of the nineteenth century, the nerves were assumed to be fused with each other in a reticular network by Camillo Golgi (1843–1926). This view was challenged by the great Spanish neuroanatomist Santiago Ramon y Cajal (1852–1934), who vigorously claimed that each neuron constitutes its own entity – the neuron doctrine. Golgi and Cajal debated this issue for decades. Ironically, these two strong antagonists shared the Nobel Prize in physiology or medicine in 1906.

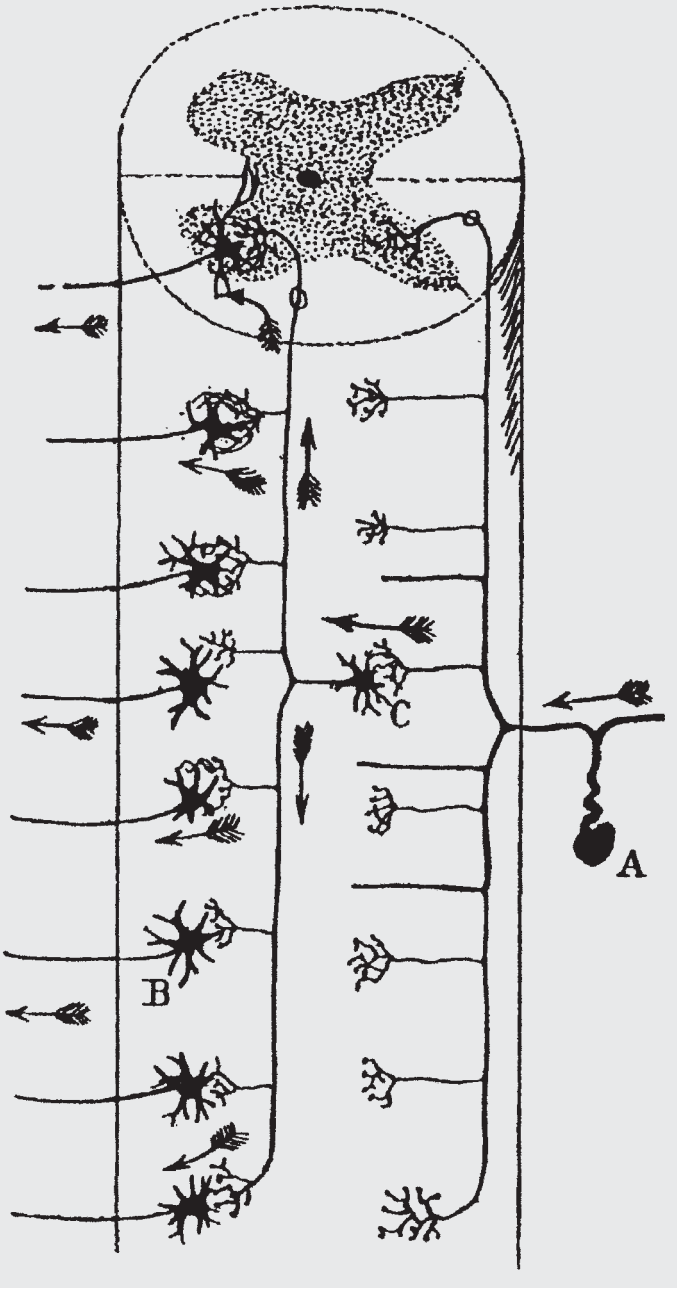

Fig. HB2 The neuron doctrine. Cajal claimed that each neuron is separate in contrast to the previously held view that the nervous system is a reticulum. (Original drawing published with permission from the Nobel Foundation.)

Golgi developed the silver impregnation method, which was subsequently named after him. However, he studied mainly the adult brain and claimed the then prevailing point of view that the nervous system is a syncytium. By studying the developing nervous system, Cajal was able to demonstrate that nerve fibers are not fused but connected via contacts. He described them as "protoplasmic kisses … the final ecstasy of an epic love story." These were later named synapses by Sherrington (see Historic box 4, p. 119).

Cajal's success was probably based on his study of embryonic material. He described his calling in the following way: "Since the full grown forest turns out to be impenetrable and indefinable, why not revert to the study of the young wood, in the nursery stage as we may say." Cajal also discovered the growth cone and stated: "One can say that the growth cone is a sort of club or battering ram, processing an exquisite chemical sensitivity, rapid ameboid movement and a certain driving force that permits it to push aside."

Cajal was born in Navarre in Spain, a son of a country doctor. He obtained his doctoral degree in Sargasso and became professor of anatomy in Valencia. It was not until he was 35 that he began to devote his life to the study of the nervous system and make his major discoveries. He was extremely productive and published 12 papers and monographs in 1889 and 16 in 1890. He published his articles in Spanish, and it took some time before they were translated into French and German and his great contributions recognized.

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Chapter 4

Neuronal migration

Sandrine Passemard, Angela M. Kaindl, Virginia Leverche, Pascal Dournaud, and Pierre Gressens

Introduction

During brain development, neocortical neurons derive from the primitive neuroepithelium and migrate to their appropriate position in the cerebral mantle, including the cortical plate (or prospective neocortex). In humans, neurons destined to the neocortex migrate mainly between the twelfth and the twenty-fourth week of gestation. In rodents, this developmental period/ontogenic step occurs roughly between embryonic day 12 (the gestation period is about 20 days in these species) and the first postnatal days. The first postmitotic neurons migrate to form a subpial pre-plate or primitive plexiform zone (for a review, see Marin-Padilla, 1998) (Fig. 4.1, p. 56). Subsequently produced neurons, which will form the cortical plate, migrate into the preplate and divide it into the superficial molecular layer (synonym layer I, marginal zone containing Cajal-Retzius neurons) and the deep subplate (synonym cortical layer VIb). Schematically, successive waves of migratory neurons pass the subplate neurons and end their migratory pathway below layer I. These migrating neurons form successively the cortical layers VIa, V, IV, II, and II, following the inside-out pattern of cortical ontogenesis discovered by the pioneering autoradiographic study of Angevine and Sidman (1961).

Migration pathways and radial glia

A central hypothesis of current developmental neurobiology (for a review, see Rakic, 1988) is that migrating neurons find their way from the germinative zone to the cortical plate by climbing along the radially ascending processes of specialized radial glial cells, a finding reported by Rakic (1971). It is, however, now largely accepted that neocortical migrating neurons originate from distinct locations and adopt di erent types of trajectory (Kriegstein & Noctor, 2004) (Fig. 4.2). Cortical pyramidal neurons migrate radially along the glia from the ventricular zone to the cortical plate. A considerable proportion of these pyramidal neurons initially adopt a tangential trajectory at the level of the ventricular or subventricular zone before following a classic radial migrating pathway along radial glia. Most or a major proportion of cortical interneurons derive from the ganglionic eminence or the ventral telencephalon in rodents and humans, respectively (Anderson *et al.*, 1997; Gleeson & Walsh, 2000). They migrate tangentially within the ventral telencephalon, and when they reach the dorsal telencephalon, they first migrate tangentially in the intermediate (prospective white matter) or subventricular zones. Subsequently, they adopt either an oblique or a radial path to reach the cortical plate. Of note, subsets of both pyramidal neurons and interneurons seem to display, at some stage of their migration, a ventricle-directed migration followed by radial movement towards the cortical plate.

Rakic (1988) has postulated that the radially arranged glial guides maintain a topographical correspondence between a hypothesized protomap present in the germinative zone and the cortical areas. The protomap hypothesis proposes that the columnar organization of the cortex arises through large numbers of neuronal precursors using a "point-to-point" radial migration from the ventricular zone to the cortical plate, i.e., through gliophilic neuronal migration. In vitro studies (Arimatsu *et al.*, 1992; Ferri & Levitt, 1993) showing an early regional specification of neuronal precursors in the absence of extrinsic stimuli support the protomap hypothesis. Integrin receptors located on radial glia and on migrating neurons seem to play a critical role in gliophilic neuronal migration (Anton *et al.*, 1999). In this context, the role of tangential migration within the germinative zone could be to permit some dispersion at the level of the cortical plate

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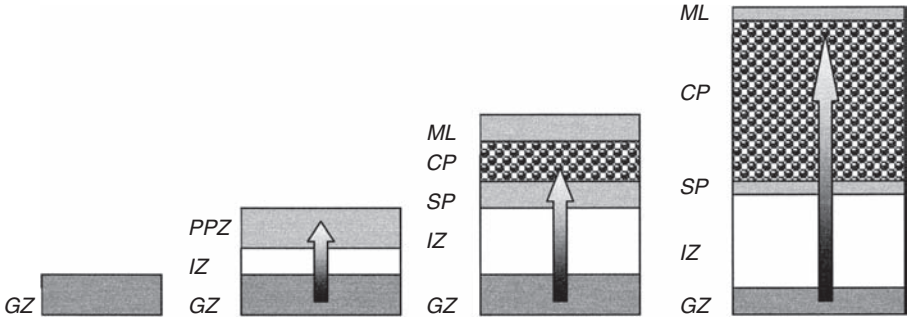

Fig. 4.1 Schematic illustration of mammalian neocortical formation. GZ, germinative zone; IZ, intermediate zone (prospective white matter); PPZ, primitive plexiform zone; SP, subplate; CP, cortical plate; ML, molecular layer. Arrows indicate migrating neurons.

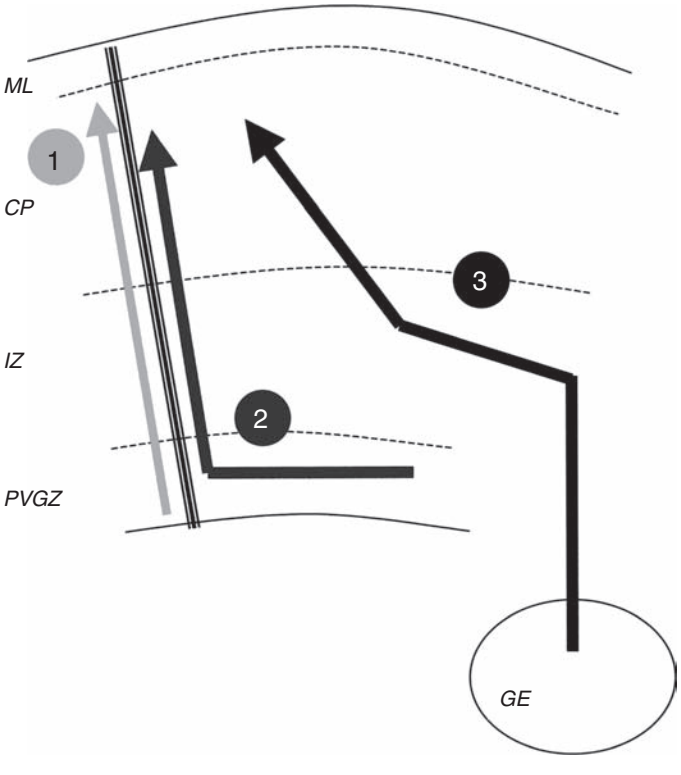

Fig. 4.2 Schematic representation of the different migratory pathways adopted by neocortical neurons from the periventricular germinative zone (PVGZ) or from the ganglia eminence (GE). 1: radial migration along glial fascicles; 2, tangential migration in the germinative zone followed by radial migration along glial guides; and 3, tangential migration in the intermediate zone (IZ). CP, cortical plate; ML, molecular layer.

of neurons originating from a single clone in the germinative neuroepithelium. This developmental mechanism would thereby increase the neuronal diversity within a given cortical area (Austin & Cepko, 1990).

Following the appearance of the cortical plate, radial glial fibers are grouped in fascicles of five to eight in the intermediate zone (Gadisseux & Evrard, 1985; Kadhim *et al.*, 1988; Gressens & Evrard, 1993). In the mouse, the first appearance of these glial units has been observed at 9.5 embryonic days (Gressens *et al.*, 1992a). The final location of neurons within the cortex, which is in part determined by the guiding glial fascicle, codetermines the connections a neuron will be able to establish. The glycogen-filled fascicles of glial fibers can also function as corridors of energy supply for migrating neurons. Along their migratory pathway, most neurons are indeed far away from the developing blood vessels that are sparse during neuronal migration. The glial fascicle therefore seems to be an ontogenic mechanism by which the mammalian brain is able to transfer neurons from the germinative zone to their distant target along a pathway that is comparatively avascular during neuronal migration (Kuban & Gilles, 1985).

The phenotype of radial glia seems to be determined both by migrating neurons (Culican *et al.*, 1990) and by intrinsic factors synthesized by glial cells. Among the latter, Götz *et al.* (1998) demonstrated that the transcription factor Pax6, which is specifically expressed by radial glia during cortical development, is critical for the morphology, number, function, and cell cycle of radial glia.

The ontogenic neuron–glia unit, composed of the radial glial fascicle and its a liated migrating neurons, is similar in mouse, rat, hamster, cat, and humans (Gressens & Evrard, 1993). Rakic (1988) has suggested that the increase in the number of symmetrical divisions of the neuronal precursors in the germinative zone could explain the evolution-linked increase of the cortical surface in mammals. We suggest a more precise and specific expansion. Since the neuron–glia unit is constant throughout the mammalian species studied, it could represent the basic module of the developing cortex: the size of the unit remains stable while the number of adjacent units gradually increases to permit brain expansion in the evolution of mammalian species. Knowledge of the genetic and environmental factors that control the organization, number, and function of these glial fascicles could, therefore, improve our understanding of cortical development and evolution of brain morphology and function.

Molecular control of neuronal migration

Studies over the past decade have identified several molecules involved in the control of neuronal migration and in targeting neurons to specific brain regions (Bielas *et al.*, 2004). These molecules can be divided into four categories (Fig. 4.3).

- *Molecules of the cytoskeleton that play an important role in the initiation and progression of neuronal movement*. Initiation-controlling molecules include filamin-A (an actin-binding protein involved in periventricular nodular heterotopia) and Arfgef2 (synonym ADP-ribosylation factor GEF2; plays a role in vesicle tra cking and is involved in periventricular heterotopia combined with microcephaly). Progression-controlling molecules include doublecortin (DCX, a microtubule-associated protein [MAP] involved in double cortex and lissencephaly), LIS1 (a MAP and dynein regulator involved in isolated type I lissencephaly and Miller–Diecker syndrome) and other molecules that are associated with migration defects in transgenic mice but which have not yet been associated with human disorders (phosphatase inhibitor 14-3-3ε, MAP1B, MAP2, and Tau).
- *Signaling molecules that play a role in lamination*. These molecules include the glycoprotein Reelin (involved in lissencephaly and cerebellar hypoplasia in humans and in the Reeler mouse mutant characterized by an inverted cortex) and other proteins generally associated with inverted cortex in transgenic or mutant mice, but which have not yet been associated with human disorders such as adaptor protein Disabled-1 (Dab1), ApoE receptor 2 (Apoer2), very-low-density lipoprotein receptor (Vldlr), two Reelin receptors, serine-threonine kinase cyclin-dependent kinase 5 (Cdk5), activator of Cdk5 p35, Brn1/Brn2, and transcriptional activators of Cdk5 and Dab1.
- *Molecules modulating glycosylation that seem to provide stop signals for migrating neurons*. These molecules include protein *O*-mannosyltransferase 1 (POMT1, associated with Walker–Warburg syndrome), protein *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase (POMGnT1, involved in muscle-eye-brain [MEB] disease), Fukutin (a putative glycosyltransferase involved in Fukuyama muscular dystrophy), and focal-adhesion kinase (Fak; involved in migration disorder in transgenic mice). These three human diseases comprise type II lissencephaly (cobblestone lissencephaly).
- *Other factors shown to modulate neuronal migration*. These include neurotransmitters (glutamate and γ-aminobutyric acid [GABA]) (Marret *et al.*, 1996; Behar *et al.*, 1999, 2001), trophic factors (brain-derived neurotrophic factor [BDNF] and thyroid hormones) (Brunstrom & Pearlman, 2000), molecules deriving from peroxisomal metabolism (Baes *et al.*, 1997; Janssen *et al.*, 2003), and environmental factors (ethanol and cocaine) (Gressens *et al.*, 1992a, b, c; Lidow, 1998).

Recent studies have highlighted the substantial cross-talk between the three first groups of molecules, but links with molecules of the fourth group have remained more elusive. Most of the recent studies have been focused on cytoskeletal and signaling molecules, with also some ongoing work on molecules conveying stop signals. Finally, a few recent studies

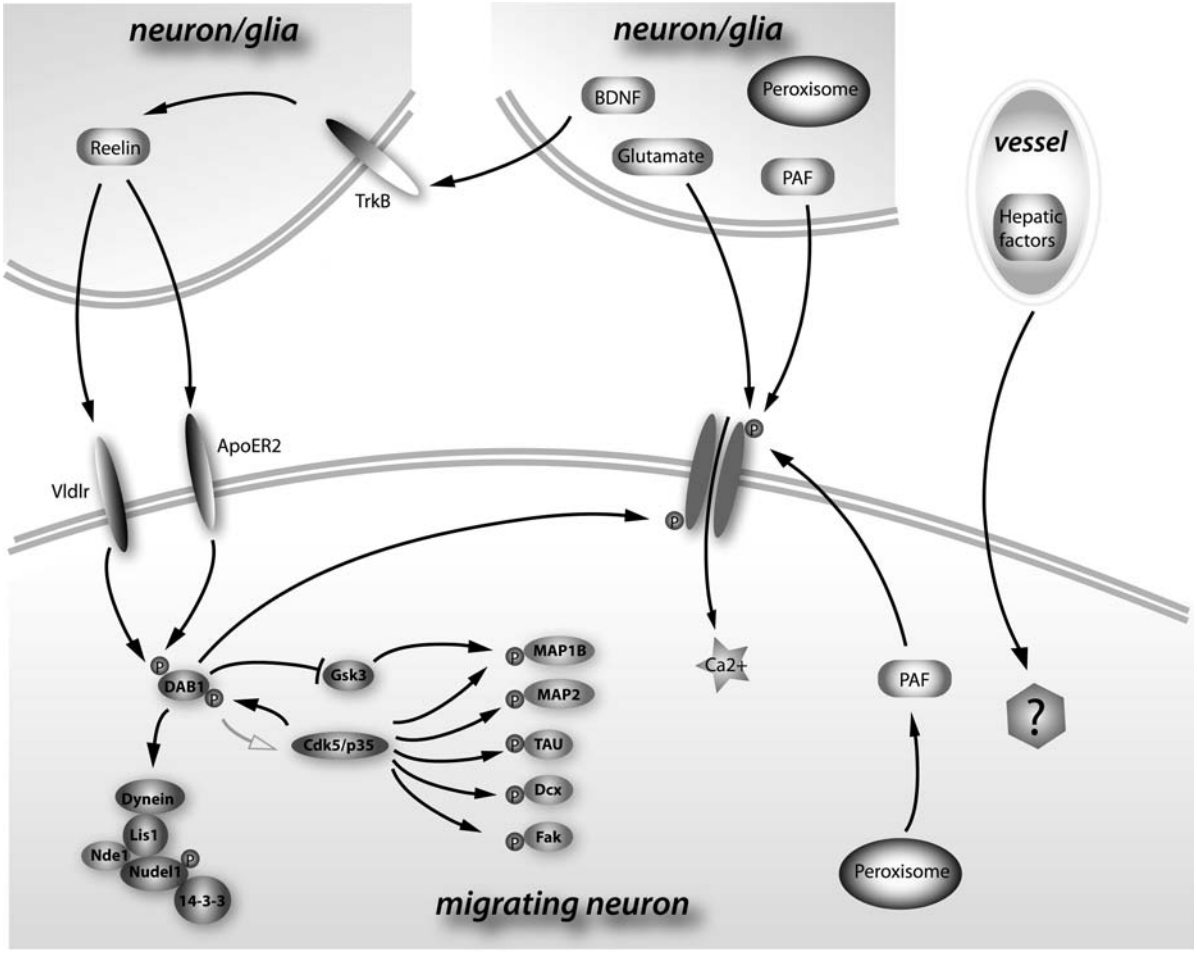

Fig. 4.3 Schematic representation of some critical molecular modulators of neuronal migration. This scheme illustrates the cross-talk between different groups of molecules, including cytoskeletal proteins, signaling molecules of the Reelin pathway, *N*-methyl-D-aspartate (NMDA) receptor-mediated pathway and peroxisome-derived factors (see text for details).

have tried to link some factors of the fourth group with more "popular" signaling molecules.

Cytoskeleton and nucleokinesis

Cytoskeletal proteins play a major role in the initiation of neuronal movement and in nucleokinesis.

Initiation of neuronal movement

Filamins are ubiquitously expressed structural proteins that cross-link the actin cytoskeleton and modulate cell response to extracellular cues by regulating changes in shape and motility (Robertson, 2005). The mechanisms by which filamins initiate neuronal migration are not yet fully understood. Filamin A probably integrates environmental cues in the ventricular zone and thereby induces changes in position, shape, and polarity of neurons through reorganization of the actin network (Nagano *et al.*, 2004; Robertson, 2005).

Nucleokinesis

Neuronal migration involves two distinct phases: extension of the leading process and movement of the cell body and nucleus (the latter is referred to as nucleokinesis) (Fox *et al.*, 1998). Movement of the centrosome (microtubule-organizing center) in the leading process precedes a movement of the nucleus towards the centrosome. During nucleokinesis, the leading process stops extending. Nucleokinesis requires the dynein/Lis1/Ndel1 complex, other cell polarity proteins (Par6), other MAPs (Dcx), signaling molecules (Cdk5, which phosphorylates Dcx and Fak), and

stop molecules (Fak). In addition, two recent studies have strongly suggested that myosin II, an actin-based protein that is localized both in the leading process of most migrating neurons and at the rear of moving neurons, is essential for cell soma movement and seems to have a negative e ect on leading process advancement (Bellion *et al.*, 2005; Schaar & McConnell, 2005). The positioning of the centrosome in the leading process and the dynamic coupling and uncoupling of the nucleus to the centrosome seem to be key events of neuronal migration.

Several recent studies have focused on Dcx function. Although Dcx is a microtubule-associated protein that is involved in X-linked double cortex syndrome, its precise role in the control of neuronal migration is not yet fully understood. Dcx could act as a molecular link between microtubule and actin cytoskeletal filaments (Bellion *et al.*, 2005; Schaar & McConnell, 2005; Tsukada *et al.*, 2005), and Dcx microtubule a nity is regulated by a balance of kinase and phosphatase activity. In addition, two other reports suggest roles of Dcx not directly related to microtubules. Dcx interacts with c-Jun N-terminal kinase (JNK) and JNK interacting protein (JIP), suggesting the involvement of Dcx in a signaling pathway (Gdalyahu *et al.*, 2004). Dcx has also been shown to interact with the ubiquitin-specific protease *Drosophila* fat facets related on X chromosome (DFFRX), which prevents degradation of di erent substrates, including β-catenin, from degradation by the proteasome, a finding that suggests a role of Dcx in cell adhesion regulation (Friocourt *et al.*, 2005).

Lis1 haplo-insu ciency is associated with type I lissencephaly (synonym classic lissencephaly; MIM#607432) and Miller–Diecker lissencephaly syndrome (MIM#247200). Lis1 protein seems to be involved in radial migration of pyramidal neurons as well as in nonradial migration of inhibitory interneurons (McManus *et al.*, 2004). Lis1 binds to dynein and dynein-associated proteins Nde1 and Ndel1, linking Lis1 with nucleokinesis. Interaction between Lis1 and dynein is a ected by binding of microtubule-associated protein 1B to Lis1 (Jimenez-Mateos *et al.*, 2005). In addition, Lis1 binds catalytic α dimers of brain cytosolic platelet-activating factor acetylhydrolase (PAFAH). PAF has been shown to enhance *N*-methyl-D-aspartate (NMDA) glutamatergic receptors currents (Tabuchi *et al.*, 1997), suggesting that, as Dcx (see above), Lis1 could control neuronal migration by a pathway not directly related to microtubule organization. Recent data suggest that Lis1 molecule undergoes complex conformation rearrangement when switching from a complex with PAFAH to the one with Ndel1 (Tarricone *et al.*, 2004).

Finally, an elegant study has shown that a loss of Lis1 causes an accumulation of multipolar progenitor cells within the subventricular zone of embryonic rat brains (Hatten, 2005; Tsai *et al.*, 2005), resulting from a failure in progression from the multipolar to the migratory bipolar state. Loss of Lis1 also induces an abolition of interkinetic nuclear oscillations of the radial progenitors in the ventricular zone and an accompanying inhibition of cell division. These results identify multiple novel roles for Lis1 in nucleokinesis and show, as previously described (McConnell & Kaznowski, 1991), a potential link between neuronal division and neuronal migration.

Signaling molecules

Signaling molecules that participate in the control of neuronal migration include the central Reelin pathway as well as less characterized pathways such as neurotrophins and thyroid hormones.

The Reelin pathway

The extracellular protein Reelin is a key for neuronal positioning during cortical development. Reelin binds to receptors including α-3 β-1 integrin, Vdrlr, and ApoER2. One major intracellular downstream e ector of Reelin is Dab1.

It has recently been shown that Reelin binds to α-3 β-1 integrin through its N-terminal region, a site distinct from binding site Reelin uses for other receptors. Also, Dab1 can form a complex with the cytoplasmic terminus of the α-3 β-1 integrin receptor in a Reelin-dependent manner (Schmid *et al.*, 2004). Mutation of *dab1* in *scrambler* mice results in abnormal positioning of cortical neurons through its regulatory role in adhesion of migrating neurons to radial glial fibers (Sanada *et al.*, 2004).

It has been hypothesized that signaling molecules link extracellular signals to cytoskeletal proteins (Bielas *et al.*, 2004). In further support of this concept, Reelin can induce phosphorylation of MAP1B, a neuron-specific MAP implicated in the control of microtubule dynamic stability and in the cross-talk between microtubules and actin. Phosphorylation of MAP1B (Gonzalez-Billault *et al.*, 2005) involves Cdk5 and glycogen synthase kinase 3 (Gsk3) (Gonzalez-Billault *et al.*, 2005).

As mentioned above, glutamate has been shown to modulate neuronal migration via NMDA receptors in vitro (Behar *et al.*, 1999) and in vivo (Marret *et al.*, 1996). In this regard, an in vivo blockade of NMDA receptors at the end of gestation in mice has been shown to disturb cortical lamination and induce clustered heterotopic neurons in layer I (Reiprich *et al.*, 2005). In a key study bridging signaling molecules and glutamate neurotransmission, Chen *et al.* (2005) showed that Reelin mediates tyrosine phosphorylation of NMDA receptors and potentiates calcium influx through these receptors in a Dab1-dependent manner.

Other pathways

A recent study has shown that neurogenin 2 (Ngn2), a proneural molecule, coordinates the acquisition in the subventricular zone of radial migration properties and dendritic unipolar morphology characteristic for pyramidal neurons (Hand *et al.*, 2005). This effect of Ngn2 requires a posttranslational phosphorylation of a specific tyrosine residue and is distinct from the proneural (see above) role of Ngn2. The effect of Ngn2 on neuronal morphology suggests some interactions with cytoskeletal molecules.

Application of BDNF or neurotrophin 4 (NT4) to the embryonic cortex induces heterotopias in the molecular layer (Brunstrom & Pearlman, 2000), a finding that suggests their participation in neuronal migration control. Using nestin-BDNF transgenic mice, a model for early increased BDNF signaling in the developing cortex, Alcantara *et al.* (2005) demonstrated recently that model-associated heterotopias result from the BDNF-induced impairment of the final radial migration of GABAergic neurons (unaltered tangential migration from the ganglionic eminence) as well as from their inability to integrate into the appropriate layer. In addition, overexpression of BDNF induces a greatly reduced expression of Reelin in the developing cortex, potentially linking the effects of neurotrophins on neuronal migration to the Reelin pathway (Alcantara *et al.*, 2005).

Thyroid hormones are known to be important for proper brain development. Early maternal hypothyroxinemia during gestation leads to irreversible brain damage and neurodevelopmental deficits. Two recent studies have highlighted the fact that experimental maternal hypothyroxinemia might impact on neuronal migration through an alteration of radial glial cell development (Martinez-Galan *et al.*, 2004) and of the tangential migration of neurons derived from the medial ganglionic eminence (Cuevas *et al.*, 2005). The mechanisms by which maternal hypothyroxinemia disrupts neuronal migration remain to be determined.

Among neurotransmitters, GABA also seems to be involved in neuronal migration modulation as it stimulates neuronal migration via calcium-dependent mechanisms in tissue culture (Behar *et al.*, 1996, 1998, 1999). Chemotactic effects of GABA were observed at femtomolar concentrations and involved the three classes of GABA receptors, whereas GABA-induced chemokinesis required micromolar concentrations and involved only $GABA_B$ and $GABA_C$ receptors. Furthermore, the relative contribution of GABA-mediated chemotaxis and chemokinesis was highly dependent on the stage of neocortical development.

Glycosylation and stop signals

The identification of the gene mutations underlying Walker–Warburg syndrome (MIM#236670; *Pomt1*) and Fukuyama congenital muscular dystrophy (MIM#253800; Fukutin gene [*FKTN*]) has paved the way for modeling these diseases in animals. Unfortunately, targeted disruption of *Pomt1* in mice leads to early embryonic death prior to onset of neuronal migration (Willer *et al.*, 2004). A preliminary report of Fukutin-deficient chimeric mice shows an early embryonic defect in basal lamina accompanied by progressive laminar disorganization (Chiyonobu *et al.*, 2005). Interestingly, the number and extent of neurons labeled with bromodeoxyuridine was not modified in chimeric embryos. These data suggest that the neuronal migration disorder is not the primary defect (Lyon *et al.*, 1993).

Peroxisomes and liver-derived factors

The lack of functional peroxisomes, as detected in the Zellweger syndrome (MIM#214100), gives rise to heterotopic neurons in the neocortex, the cerebellum, and the inferior olivary complex. Animal models of this disease have been established by inactivation of a gene critically involved in peroxisomal assembly (Baes *et al.*, 1997). Analysis of these models showed that: (i) the migration defect was partially caused by altered NMDA receptor-mediated calcium mobilization; (ii) this NMDA receptor dysfunction was linked to a deficit in the ether lipid PAF synthesis; and (iii) normal neocortex development requires normal peroxisomal metabolism not only in the brain but also in the liver. In addition, recent data support the hypothesis that absence of peroxisomes in neural cells induces a delay in

neuronal migration while absence of peroxisomes in the liver induces a partial arrest of neuronal migration (Krysko *et al.*, 2007). Together, these data highlight the cross-talk between liver and brain and identify distinct roles for hepatic and brain peroxisome-derived factors in the control of neuronal migration.

Lissencephalies: classification

The term lissencephaly (literally "smooth brain") covers rare malformations that have in common a reduction of cortical gyration and abnormal cortical layering. Di erent forms of lissencephaly have been described, and there is still no final consensus on their classification. In this chapter we have adopted a classification based on those proposed by Barkovich and colleagues (2005) and Sarnat and Flores-Sarnat (2003), which take their etiologies and associated malformations into account (Table 4.1). This classification distinguishes two major families: classic lissencephalies (also called type I) and its variants and cobblestone lissencephalies (also called type II). Four types of classic lissencephaly have been described according to the genetic etiology: abnormalities in genes *LIS1*, *DCX*, *TUBA3*, or *ARX*. In addition, classic lissencephalies also include isolated lissencephalies without any identified genetic defect, lissencephalies with severe microcephaly (microlissencephaly) and lissencephalies associated with syndromes of multiple malformations.

Table 4.1 Classi cation of lissencephalies

(A) Classic lissencephalies and variants
Classic lissencephalies
– *LIS1* gene mutations
– *DCX* gene mutations
– *TUBA3* gene mutations
Type 1 unexplained lissencephalies
ARX gene mutation with agenesis of the corpus callosum (XLAG syndrome)
Lissencephalies with cerebellar hypoplasia (LCH)
Microlissencephalies
Syndromic lissencephalies
(B) Cobblestone lissencephalies
Walker–Warburg syndrome
Fukuyama syndrome
MEB disease
(C) Type III lissencephalies

The term variant refers to the presence of a corpus callosum agenesis and/or of a cerebellar hypoplasia. Cobblestone lissencephaly results from a global disorganization of brain organogenesis and, in particular, from an abnormal glia limitans leading to complex migratory disorders. Cobblestone lissencephaly is essentially observed in the three related syndromes: Walker–Warburg, Fukuyama, and MEB disease (MIM#253280). Finally, some forms of lissencephaly such as type III lissencephaly (MIM#611603) do not fit into any of these categories.

Classic lissencephalies (type I lissencephalies)

Classic lissencephalies form a genetically heterogeneous group with highly variable neuroradiological signs (Barkovich *et al.*, 2001; Fig. 4.4). Complete agyria (lack of gyration) has to be distinguished from pachygyria (incomplete gyration with a reduced number of at and broad gyri separated by shallow sulci). The shallow sylvian fissures result in a figure-of-eight appearance of the axial brain sections. Aspect and severity can vary according to the cortical area and generally follow a rostrocaudal gradient. Hippocampus and temporal cortex are often less a ected. Dobyns and Truwit (1995) have proposed a radiological score of severity based on the presence and location of agyria, pachygyria, and subcortical band heterotopias. On MRI, the cortex appears thickened: 5–20 mm, whereas the normal thickness is between 2.5 mm and 4 mm. The microscopic cytoarchitecture is abnormal (reduction of the number of cortical layers and abnormal neuronal densities), yielding a neuropathological pattern that is specific for each molecular abnormality described so far. Lateral ventricles can be enlarged in their posterior portion (colpocephaly).

Lissencephaly variants are characterized by major abnormalities of the corpus callosum and of the cerebellum. However, this distinction is not absolute: if the cortical malformation is the key feature in classic lissencephalies, minor abnormalities of the corpus callosum (rostral hypoplasia or partial agenesis) or vermis hypoplasia can be observed especially with *DCX* mutations. Conversely, cerebral malformations are not constant in X-linked lissencephaly with ambiguous genitalia syndrome (XLAG syndrome or X-linked lissencephaly type 2, MIM#300215). By definition, head circumference is above –3 standard deviations (SD) in classic lissencephalies while it is below –3 SD in microlissencephalies. The incidence

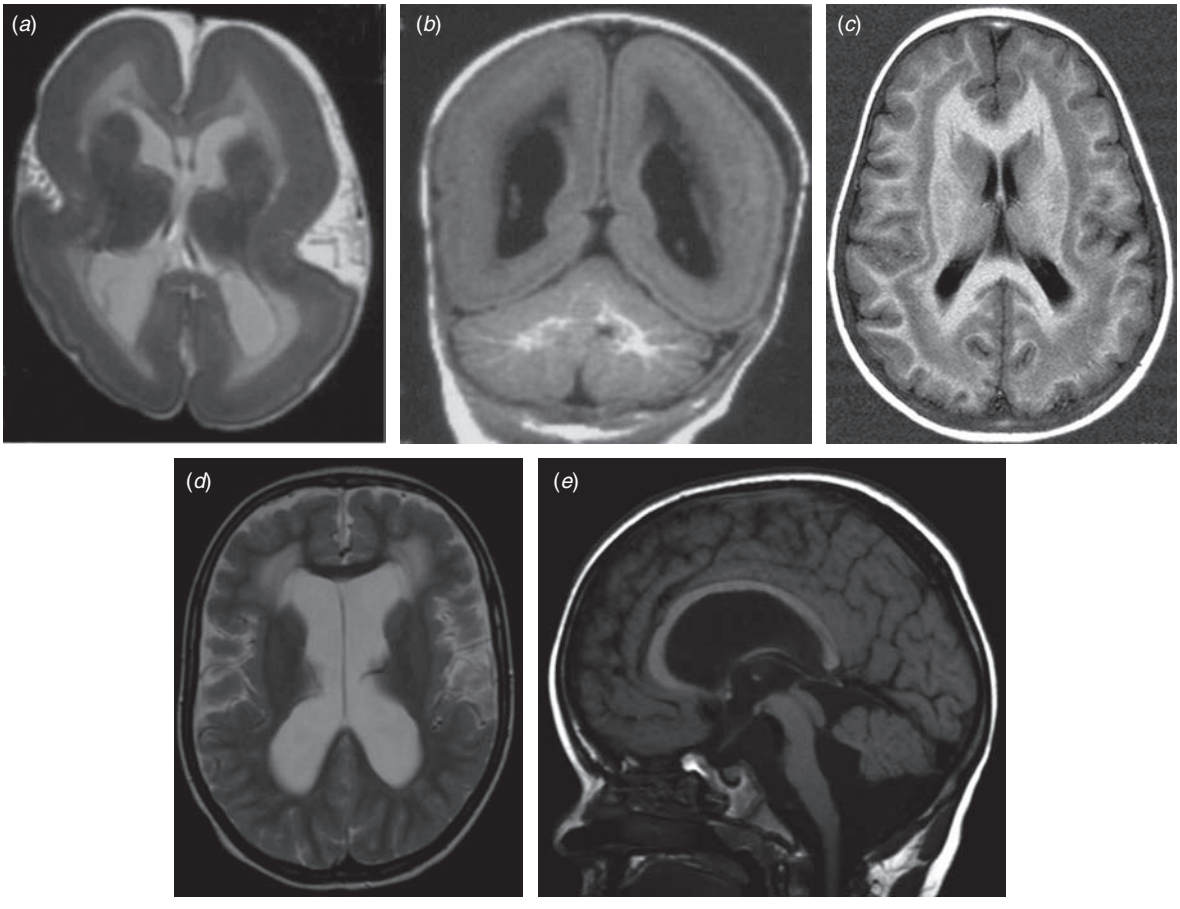

Fig. 4.4 (*a*, *b*) Classic lissencephaly (*LIS1* gene mutation). T2-weighted brain magnetic resonance imaging (MRI; axial section) (*a*) showing agyria-pachygyria with anteroposterior gradient (anterior being more severe) and the classical gure-of-eight appearance of shallow sylvian ssures. T1-weighted brain MRI (coronal brain section) (*b*) showing the preserved cerebellum. (*c*) Classic X-linked lissencephaly caused by doublecortin (*DCX*) gene mutation. T1-weighted brain MRI (axial section) showing the subcortical laminar heterotopia with a thickened cortex and a rostrocaudal gradient. (*d*, *e*) Cobblestone lissencephaly. T2-weighted brain MRI (axial section) (*d*) showing pachygyria and polymicrogyria with anteroposterior gradient, and hydrocephalus. T1-weighted brain MRI (sagittal section) (*e*) showing brainstem and cerebellar hypoplasia.

of classic lissencephaly is estimated to be 1.2 cases/100 000 live-births.

LIS1 mutations (isolated lissencephaly and Miller–Dieker syndrome)

Abnormalities of the *LIS1* gene (synonym platelet-activating factor acetylhydrolase isoform 1B α subunit gene PAFAH1B1; MIM#601545), which encodes the LIS1 (synonym PAFAH1B1) protein, explain the pathology seen in 40% of patients with lissencephaly (Reiner *et al*., 1993). Deletions and nonsense mutations of *LIS1* induce an agyric-pachygyric phenotype with a postero-anterior gradient (posterior being more severe; grade 2–3 according to Dobyns). Missense mutations can induce less severe phenotypes (grade 4 according to Dobyns). Neuronal heterotopia and cerebellar hypoplasia can be observed (lissencephaly with cerebellar hypoplasia [LCH] type A, see below). In exceptional cases, band heterotopia can be observed in cases with *LIS1* mutation. The cortex is thickened (10–20 mm), disorganized and is generally composed of four layers: a large molecular layer, a layer of superficial neurons, a paucicellular layer containing myelinated fibers, and a deep layer containing neurons that failed to reach their final target. The 17p13 deletion that contains *LIS1* gene is responsible for the Miller–Dieker syndrome (Kuwano *et al*., 1991), which combines a grade 1 lissencephaly and dysmorphic features (prominent forehead, bitemporal hollowing, micrognathia, malpositioned and/or malformed ears, short nose with upturned nares and low nasal bridge, long and thin

upper lip, and delayed tooth eruption). Septal calcifications are sometimes observed.

DCX mutations

The *DCX* or *XLIS* gene, encoding doublecortin or DCX and located on the X chromosome, is responsible for the disease in about 40% of patients with lissencephaly and in 85% of patients with subcortical laminar heterotopia (des Portes *et al.*, 1998). In boys, mutations induce a classic lissencephaly (grade 1 to grade 4) with a thickened cortex (10–20 mm) and a rostrocaudal gradient, i.e., a gradient reverse to that observed in patients with *LIS1* mutations. Heterozygous girls have subcortical laminar heterotopia, which consists of a layer of gray matter of variable thickness located between the normally located superficial cortical gray matter and the lateral ventricle ("double cortex"). This double cortex may be limited to the anterior part of the hemispheres. The clinical phenotype in females is variable and ranges from a complete lack of neurological signs to mental retardation and epilepsy. Female carriers do not have a preferential X chromosome inactivation and, therefore, a normal MRI finding does not exclude the presence of a carrier status.

In neuropathological studies, brains from patients with *LIS1* mutations exhibited the classic inverted four-layer lissencephalic architecture and unique cytoarchitectural findings, including a roughly ordered six-layer lamination in male patients with *DCX* mutations and lissencephaly.

TUBA3 mutations

α-3 tubulin (*TUBA3*) gene mutations have been described recently (Keays *et al.*, 2007) in two male/female individuals. The first patient presented with a classic lissencephaly with a thick disorganized cortex and, clinically, with severe epilepsy, mental retardation, and motor deficits. The second patient exhibited less severe cortical abnormalities, with temporal and rolandic pachygyria and abnormal organization of the hippocampus. Both patients had corpus callosum agenesis, inferior vermis abnormalities, and brainstem hypoplasia.

The *TUBA3* gene is the human homolog of the murine *Tuba1* which is expressed during early embryonic development. *Tuba1* mutations impair the ability of the protein to bind guanosine triphosphate (GTP) and to form native heterodimers with β-tubulin, which is very important for microtubule function. Mutation in murine *Tuba1* induces abnormal neuronal migration with disturbances in layers II/III and IV of the visual, auditory, and somatosensory cortices.

ARX mutations

ARX (aristales-related homeobox) gene mutations cause a particular classic X-linked lissencephaly with or without corpus callosum agenesis. The cortex in three layers of ARX lissencephalies is less thick (5–10 mm) than in the other classic lissencephalies and the cortical abnormality displays a rostrocaudal gradient (frontal regions being more a ected) (Kato & Dobyns, 2005). Female carriers can have isolated or combined corpus callosum agenesis, epilepsy, and/or mental retardation.

XLAG syndrome (Berry-Kravis & Israel, 1994) is also associated with mutations of the *ARX* gene. A ected boys have lissencephaly, corpus callosum agenesis, facial dysmorphic features, ambiguous genitalia, problems with thermoregulation, and severe epilepsy, which can begin prenatally.

ARX gene mutations are implicated in a wide spectrum of X-linked disorders extending from mild forms of X-linked mental retardation without apparent brain abnormalities to severe lissencephaly. Phenotypes include corpus callosum agenesis with mental retardation, X-linked West syndrome, and Partington syndrome (hand dystonia). A phenotype–genotype correlation has not been established.

The *ARX* gene is expressed mainly in telencephalic structures. In adults, *ARX* gene expression becomes restricted to a population of GABAergic neurons (Poirier *et al.*, 2004). ARX seems to be implicated in interneuron migration from the ganglionic eminence (future thalamus), however its role in cell di erentiation and neuronal migration needs to be further clarified.

Lissencephaly with cerebellar hypoplasia

Lissencephaly with cerebellar hypoplasia (LCH) is a heterogeneous group of lissencephalies in which there is cerebellar hypoplasia preferentially of the vermis and hemispheres that display sulci. A temporary classification comprising eight subtypes has been proposed (Ross *et al.*, 2001). While the phenotype of type A LCH is caused by *LIS1* or *DCX* gene mutations (MIM#607432, MIM*300121), patients with type B LCH have/display *RELN* gene mutations (MIM*600514). The latter lissencephaly is more severe than type A LCH but

has a rostrocaudal gradient similar to that seen in patients with *DXC* gene mutations. The cortex is quite thick (5–10 mm), whereas the cerebellum is hypoplastic and smooth.

Genes responsible for other forms of LCH are not known. Type C LCH patients usually have a cleft palate. Type D LCH patients (synonym Barth syndrome; see below) display a neuropathology that includes massive brain, cerebellum and corticospinal tract hypoplasia; their cortex is very thick (10–20 mm). Type E LCH is close to type A LCH, but with a marked gradient from frontal agyria to occipital pachygyria. Type F LCH includes corpus callosum agenesis. Moreover, two new types of LHC have been reported: LCH with corpus callosum agenesis and cerebellar dysplasia (Miyata *et al.*, 2004) and a subtype with cerebellar hypoplasia, Dandy–Walker malformation, and myoclonic epilepsy.

Microlissencephaly

Microlissencephaly di ers from other lissencephalies by a severe microcephaly (head circumference below –3 SD). This suggests a neural stem cell proliferation defect and/or an increase in apoptosis process in addition to migration defects. There are two types of microlissencephaly: type A, also referred to as Norman–Roberts lissencephaly syndrome, without infratentorial findings; and type B, also referred to as Barth syndrome (MIM#302060), which includes a massive cerebral, cerebellum, and corticospinal tract hypoplasia. In the latter disease, patients also display dilated cardiomyopathy, neutropenia, myopathy, and abnormal mitochondria. Microlissencephaly needs to be distinguished from microcephaly with simplified gyral pattern (MIM#603802) but with a normal six-layer cortex.

The autosomal recessive inherited Norman–Roberts syndrome is characterized by severe microcephaly, epilepsy, severe mental retardation, short stature, and dysmorphic features. This lissencephaly could be identical to LCH type B and has therefore been linked to *RELN* gene mutations.

Syndromic lissencephaly

Two types of lissencephalies are included in this group: lissencephalies associated with other neurological abnormalities (such as lissencephaly/pachygyria associated with peripheral demyelinating axonopathy) and lissencephalies observed in syndromes of multiple malformations (in which lissencephaly is generally inconstant and not necessary for diagnosis). Among these syndromes, craniotelencephalic dysplasia (MIM#218670; extensive craniosynostosis, microphthalmia, encephalocele), Warburg micro syndrome (MIM#600118; corpus callosum agenesis, microphthalmia, microcephaly, cataract, dysmorphic features), Goldenhar syndrome (synonym hemifacial microsomia, MIM#164210; branchial arch development abnormalities, hemifacial microsomia, microtia) or Baraitser–Winter syndrome (MIM#243310; hypertelorism, ptosis, coloboma, pachygyria/lissencephaly with a frontal predominance) have been reported.

Cobblestone lissencephalies (type II lissencephalies)

Cobblestone lissencephalies are characterized by a pachygyric or granular brain appearance with shallow sulci and hypomyelination with subcortical cystic cavitations. Lateral and third ventricle dilatation, which can be severe, and vermis hypoplasia or general cerebellar and brainstem hypoplasia with small pyramids can be observed. Additional brain anomalies such as hypoplasia/agenesis of corpus callosum, occipital encephalocele, and Dandy–Walker malformation have been described. The hemispheres can be merged in the midline by gliosis. The cortex is thickened (7–10 mm), disorganized, and invaded by gliovascular fascicles. White matter contains many heterotopic neurons. Typically, the brain is surrounded by a neurofibroglial envelope; this envelope is not observed in classic lissencephalies and gives a bumpy (hence "cobblestone") rather than smooth aspect to the cortical surface. The cortical development defect most likely occurs between 6 and 24 weeks of gestation. Many neurons migrate too far through a defective glial-limiting membrane into the subpial space, i.e., beyond the cortical plate. Cobblestone lissencephalies are linked to abnormal *O*-glycosylation of α-dextroglycan (Martin, 2005). Dextroglycan gene (*DG1*) encodes a precursor protein that is cleaved into α and β DG1. Dextroglycan is a transmembrane polypeptidic complex that bridges dystrophin with extracellular laminin. *POMT1* (9q34 locus) and *POMT2* encode the two *O*-mannosyltransferase proteins 1 and 2 (POMT1, POMT2) that need to form a complex in order to catalyze the first step of *O*-mannosylation of β dextroglycan. *POMGnT1* encodes an *O*-mannosyl-β-1,2-*N*-acetylglucosaminyltransferase, which transfers an *N*-acetylglucosamine residue to an *O*-linked mannose. The *FCMD* gene on chromosome 9q31 (synonym

FKTN) encodes the fukutin protein, and the *FKRP* gene encodes a protein with a sequence close to that of fukutin. Based on their sequence, these two proteins are thought to be Golgi glycosyltransferases. However, their precise functions are still unknown.

Clinically, cobblestone lissencephalies are observed in three autosomal recessive syndromes, which have been shown to display a considerable overlap on the basis of recent molecular genetic discoveries. The incidence of cobblestone lissencephalies is not know, but is most likely around 1 in 100 000 live-births.

Walker–Warburg syndrome

Walker–Warburg syndrome is the most common and the most severe form of cobblestone lissencephaly. It is characterized by the combination of brain malformations such as hydrocephalus, agyria, retinal dysplasia, and sometimes occipital encephalocele (HARD ± E syndrome) with structural eye abnormalities and muscular dystrophy. Newborns die within the first postnatal months. Eye abnormalities include cataracts, microcornea and microphthalmia, retinal dysplasia, hypoplasia or atrophy of the optic nerve, and glaucoma. In 30% of patients, this phenotype is linked to *POMT1* (Beltran-Valero de Bernabe *et al.*, 2002) or *POMT2* (van Reeuwijk *et al.*, 2005) mutations. Mutations in *FCMD* or *FRKP* genes have been exceptionally reported. In addition, *POMT1* mutations can cause muscular dystrophy such as congenital muscular dystrophy plus mental retardation and limb-girdle muscular dystrophy type 2K (LGMD2K, MIM#609308) associated with mental retardation and microcephaly (van Reeuwijk *et al.*, 2006).

Fukuyama syndrome and muscle-eye-brain disease

The severity of the brain involvement in Fukuyama syndrome is milder than that in Walker–Warburg syndrome and MEB disease, and the eyes are only occasionally a ected severely. MEB disease is characterized by eye involvement (congenital myopia and glaucoma, retinal hypoplasia), mental retardation, and structural brain involvement (pachygyria, at brainstem, and cerebellar hypoplasia).

Fukuyama disease is linked to mutations of the *FCDM* gene (Kobayashi *et al.*, 1998). MEB disease is linked to *POMGnT1* (1p34-p33 locus) (Yoshida *et al.*, 2001) and less frequently to *FRKP* (19q13 locus) mutations (Beltran-Valero de Bernabe *et al.*, 2004). As for *POMT1* mutation-induced phenotypes, cobblestone lissencephaly is not the only clinical feature, but has been reported in congenital muscular dystrophy 1C (MDC1C, MIM#606612) and LGMD2I (MIM#607155).

Other cobblestone lissencephalies

Cobblestone lissencephalies without muscular dystrophy have been reported. Some of them are associated with retinal abnormalities or severe myopia. It is not clear whether these lissencephaly subtypes are linked to Walker–Warburg or Fukuyama syndromes or MEB disease. Dobyns described a lissencephaly associated with corpus callosum agenesis, trigonocephaly, and coloboma microphthalmia (Ramer *et al.*, 1995). Another type of lissencephaly with arthrogryposis, deafness, and muscular dystrophy without creatine kinase increase has also been reported (Seidahmed *et al.*, 1996).

Type III lissencephaly

Type III lissencephaly is characterized by severe microcephaly, agyria, corpus callosum agenesis, cerebellar, basal ganglia hypoplasia, a thin six-layered cortex, and blurred white matter borders. This lissencephaly has been reported in three autosomal recessive syndromes: Neu–Laxova syndrome (MIM#256520), Enchara–Razavi–Larroche lissencephaly (synonym Lissencephaly type III and bone dysplasia, MIM#601160; microcephaly, corpus callosum agenesis, cystic cerebellum brainstem hypoplasia and fetal akinesia [Encha Razavi *et al.*, 1996]) and a syndrome, reported by Plauchu, with lissencephaly, severe microcephaly, corpus callosum agenesis, cerebellar hypoplasia, dysmorphic features, and punctuate epiphysis. Neuropathological findings are very similar in these three entities and thereby suggest that they are either allelic diseases or linked to genes implicated in the same function or pathway (Allias *et al.*, 2004).

X-linked periventricular heterotopia

The human X-linked dominant periventricular heterotopia (MIM#300049) is characterized by neuronal nodules lining the ventricular surface. Hemizygous-a ected males die within the embryonic period, and a ected females have epilepsy that can be accompanied by other manifestations such as patent ductus arteriosus and a coagulopathy. The gene responsible for this disease, filamin-A gene (*FLNA* [Nagano *et al.*, 2004]), encodes an actin cross-linking phosphoprotein, which transduces ligand–receptor binding into actin

reorganization. Filamin A is necessary for locomotion of several cell types and is present at high levels in the developing neocortex. Rakic and colleagues (1996) have shown previously the polarity of microtubule assemblies during migration of rodent cerebellar neurons and proposed a critical role of the dynamics of slow polymerization combined with fast disintegration of oriented microtubules for the displacement of the nucleus and cytoplasm within the membrane cylinder of the leading process of migrating neurons. On the other hand, microfilaments seem important for the leading edge extension.

Null mutations in *FLNA* (MIM#300017) induce, through a loss-of-function mechanism, X-linked periventricular heterotopias and extraneural abnormalities (cardiac valvular anomalies, propensity to premature stroke, small joint hyperextensibility, gut dysmotility, and persistent ductus arteriosus) (Fox *et al.*, 1998). This indicates a key role of filamin A in vascular function and in connective tissue integrity. In contrast, missense mutations in the same gene induce, through a gain-of-function mechanism, a spectrum of malformations in multiple organs predominantly of the skeleton (Robertson, 2005). The precise molecular mechanisms underlying such distinct phenotypes remain to be determined. Also, the discrepancy between the ubiquitous expression of filamins and the rather discrete phenotype associated with null mutations warrant further investigation.

Zellweger syndrome

The Zellweger cerebrohepatorenal syndrome (MIM#214100) is a fatal autosomal recessive disease caused by an absence of functional peroxisomes. One hallmark of this human disease is the presence of heterotopic neurons in the neocortex, the cerebellum, and the inferior olivary complex. A ected neonates display severely retarded and/or rapid regression of psychomotor development, facial dysmorphism, and severe muscular hypotonia; they usually die within the first postnatal months.

Environmental effects on neuronal migration

Neuronal migration disorders have been described in humans and/or in animal models following exposure to several environmental factors in utero, including infection with cytomegalovirus or toxoplasmosis, ethanol, cocaine, or ionizing radiation. In most cases, the mechanisms by which these factors disturb neuronal migration remain unclear.

Cocaine exposure during gestation has been shown to disturb neuronal migration and cortical addressing both in mice and monkeys (Gressens *et al.*, 1992a, b; Lidow, 1998). Cocaine exposure in mice was recently shown to specifically decrease GABA neuron migration from the ganglionic eminence to the cerebral cortex but not to the olfactory bulbs, suggesting a degree of specificity in the e ects of cocaine on neuronal migration (Crandall *et al.*, 2004).

In the human fetal alcohol syndrome, neuronal molecular ectopia has been described in several cases although this sign is not restricted to this syndrome (Kaindl *et al.*, 2006). Animal studies have identified abnormalities of radial glia and disturbances of transformation of radial glia into astrocytes (Guerri *et al.*, 2001).

Conclusion

Compelling evidence points to the central role of cytoskeletal proteins and the Reelin pathway in the control of neuronal migration and thus in the pathophysiology of neuronal migration disorders. Future studies will need to decipher further the molecular mechanisms of action and cross-talk of these proteins.

Despite the major advances mentioned above, several questions will probably deserve some attention and research e ort. What is the real implication of other factors such as glutamate receptors or trophic factors in neuronal migration disorders in humans? What is the impact of environmental factors (including factors external to the pregnant woman, factors deriving from the maternal body, factors deriving from extraneural fetal tissues such as the liver) on neuronal migration? What is the modulatory e ect of these environmental factors on the anatomical and clinical phenotype of genetically induced migration disorders? What is the impact of abnormal migration on connectivity? New imaging techniques such as diffusion tensor imaging seem to be promising with regard to addressing this last question in humans as recently shown in type I lissencephaly (Rollins *et al.*, 2005) and peroxisomal disorders (ter Rahe *et al.*, 2004).

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Chapter 5

The neonatal synaptic big bang

Jean-Pierre Bourgeois

Introduction

Making one synapse

Each synaptic contact is a highly complex molecular machine that extracts and integrates the numerous signals circulating among the neurons (neurotransmitters, neuromodulators, trophic factors, hormones, ions, larges families of intercellular adhesion molecules, etc.), within each neuron (the multiple and diverse cascades of intracellular signaling pathways), and along each neuron (action potentials, electrotonic depolarizations, etc.). Synapses are involved in selecting and maintaining traces of the pertinent signals required for an individual's cognitive performances. It takes 30–120 minutes to build one complete synapse between two neurons cultivated in vitro (Lohmann & Bonhoeffer, 2008). Hundreds (or thousands?) of distinct classes of molecules as diverse as cytoskeletal proteins, cell adhesion molecules, ion channels, receptors, phosphatases, phosphorylases, glycolipids, and calcium-binding proteins are assembled into multiple, highly organized scaffoldings in the presynaptic and postsynaptic compartments, and in the synaptic cleft (Samuels *et al.*, 2007). They ensure e cient, fluctuating synaptic neurotransmission (timescale – milliseconds) and the structural stability of the mature synapse (timescale – months or years). The low a nities of these assembled proteins, as well as the mobility of the pharmacological receptors (Levi *et al.*, 2008) also allow the molecular reorganizations associated with synaptic plasticity (timescale – seconds to weeks). These mechanisms, which ensure both stability and plasticity of the synapse, may be dependent on the high metabolic activity observed in human brain imaging.

Each synapse is a structural and functional point of articulation between the two sets of constraints presented by the genome and the environment. Intracellular signaling pathways relay information about permanent changes in the external environment to the highly dynamic patterns of gene expression, so that neurons can respond appropriately. Synaptogenesis is controlled by genes and modulated by the numerous classes of diffusible molecules mentioned above. In addition, each synapse is distinct according to its topological position on the neuron and the neuronal phenotype. Each synapse can also have many distinct functional and morphological states, depending on the functional level of the neuronal circuits in which it is involved at a particular time (Bourgeois, 2005). The elimination of a synapse is also an active microphysiological process involving the complement cascade (Stevens *et al.*, 2007). The above discussion describes the making of a single synapse. Throughout development and in adult life, the human cortex makes and eliminates billions of synapses.

Making billions of synapses

In the adult mouse, a cubic millimeter of cerebral cortex contains about 90 000 neurons, 3 km of tiny axonal branches, 450 m of dendritic branches, and 300–900 million synaptic contacts (Braitenberg & Schüz, 1998). In addition to these are the glial cellular processes. Similar numbers are found in the human cerebral cortex. These densely packed networks of cellular processes, constituting the cortical neuropil, are actually less noteworthy than is the precision of their geometrical organization. Along with their highly organized forms, synaptic contacts are distributed in specific patterns (White, 2007). We can sum up this in a single word: synaptoarchitectony. According to their neuronal phenotypes and/or the mammalian species, axonal and dendritic arborizations can be either overlapping or excluding (Millard & Zipursky, 2008).

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Corticogenesis involves three major histological events. The first of these is neurogenesis. The future neurons are generated in the germinative ventricular layer of the telencephalic neuroepithelium very early in embryonic life, between 40 and 100 days after conception in the macaque, and between 42 and 120 days after conception in the human cerebral cortex (Rakic, 2007). The newly generated neuroblasts migrate long distances through the fetal cortical neuroepithelium to reach their final position in the cortical plate of the fetal cortex (for definitions, see Rakic, 2007, and also Chapter 4, Fig. 4.1). The second event is "hodogenesis" (this term encompasses the growth of intra- and interhemispheric axonal pathways), which starts as soon as the postmitotic neuroblasts leave the germinative ventricular zone and migrate toward the fetal cortex. These newly generated neuroblasts become polarized and produce axons that navigate toward their specific cortical or subcortical targets according to their own combinations of genetic expression (Leone *et al.*, 2008). The third event is synaptogenesis. When axonal and dendritic branches meet and are cytologically compatible, they rapidly form ensembles of synaptic contacts. This event is the topic of the present chapter.

Kinetics of synaptogenesis

Neuroanatomical investigations of the human cerebral cortex have tended to rely on postmortem histological samples. However, the parsimonious data obtained from these studies are not as precise as those obtained by using laboratory animals prepared under controlled experimental conditions. Data obtained from nonhuman primates have helped us to interpret the rare data obtained from human cortex. Using quantitative electron microscopy, we first delineated the kinetics of synaptogenesis in several cortical areas of the macaque monkey (*Macaca mulatta*), from conception to death (Rakic *et al.*, 1986; Zecevic *et al.*, 1989; Zecevic & Rakic, 1991; Bourgeois & Rakic, 1993; Bourgeois *et al.*, 1994; Granger *et al.*, 1995). In cerebral cortices of nonhuman and human primates, the kinetics of

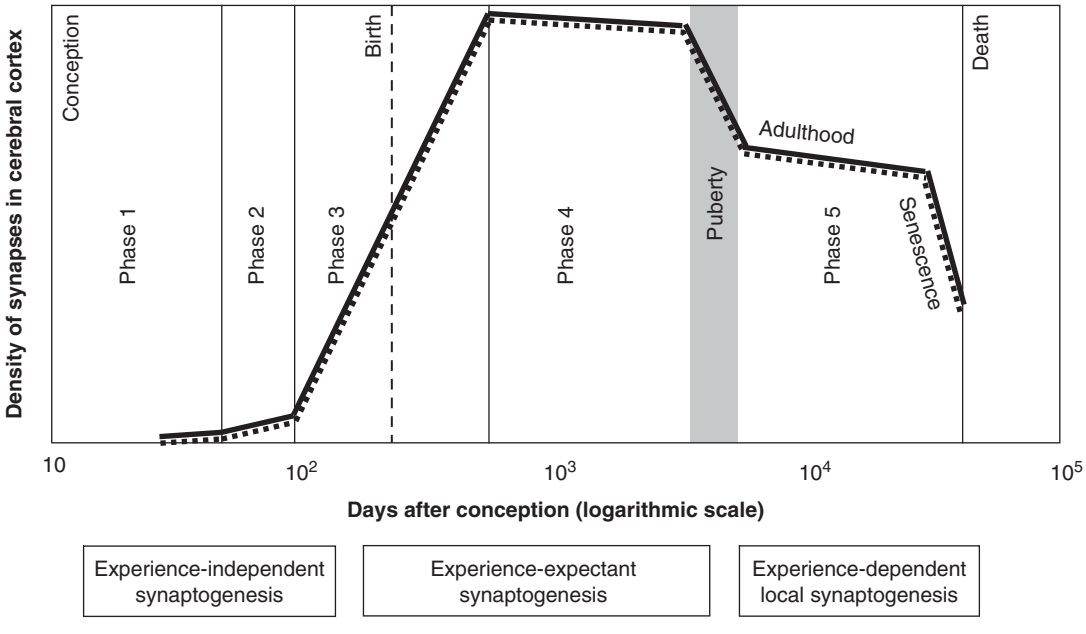

Fig. 5.1 Schematic representation of the kinetics of synaptogenesis in primary visual and prefrontal cortices of macaque monkey. The density of synaptic contacts (cartesian *y* axis) is presented as a function of the number of days after conception (logarithmic *x* axis). Similar kinetics are observed in the primary visual (solid line) and prefrontal (dotted line) cortices. Synapses appear very early in fetal life. Five distinct phases are identified here according to the tempo and amplitude of synaptogenesis. During phases 1 and 2, very early synaptogenesis occurs in the protocortex with a slow tempo and at low density. The spontaneous activity contributes to the wiring of these early synapses. The environment-related evoked activity is not yet present: synaptogenesis is independent of experience. Phase 3 begins two months before birth and ends two months after birth. In this phase, there is an "explosion" in the production of synapses. During phase 4, "the plateau phase," the density of synapses is maintained at a maximal density of synapses until the onset of puberty. This is a critical period of development when synaptogenesis becomes experience-expectant. During puberty a "synaptic catastrophe" occurs, with considerable loss of synapses. The steady-state phase 5 displays no noteworthy decrease in the mean density of synapses, until senescence. The large interindividual variability is not illustrated here.

synaptogenesis appear to be a highly complex, nonlinear, and protracted developmental process. We have identified five distinct phases of synaptogenesis, as described below.

The precocious life of synapses

Phase 1

In the cerebral cortex of the macaque, the first synapses are observed very early in embryonic life, at about embryonic day (E)50–60, possibly earlier (Rakic *et al.*, 1986; Zecevic *et al.*, 1989; Zecevic & Rakic, 1991; Bourgeois & Rakic, 1993; Bourgeois *et al.*, 1994; Granger *et al.*, 1995). Based the observations of Zecevic (1998) on the human occipital cortex, it is tentatively proposed that phase 1 might begin around six to eight weeks of gestation. In both human and macaque species this early synaptogenesis occurs at a low density and there is a low rate of accumulation of synapses in the marginal zone and intermediate zone of the fetal white matter, but not yet in the cortical plate (for an explanation of this terminology, see Chapter 4, Fig. 4.1, p. 56).

Phase 2

This second early phase of synaptogenesis takes place in the cortical plate itself, at E70–100 of the macaque embryonic life (Rakic *et al.*, 1986; Zecevic *et al.*, 1989; Zecevic & Rakic, 1991; Bourgeois & Rakic, 1993; Bourgeois *et al.*, 1994; Granger *et al.*, 1995). In the human cerebral cortex, this phase begins around 12–17 weeks of gestation (Zecevic, 1998). The synaptogenesis follows an inside-out gradient of density similar to the gradient of neuronal migration and penetration and growth of axons toward the pial surface. The first synaptic contacts are formed on the few dendritic shafts present at that time.

Phase 3: The synaptic "big bang" in the newborn brain

Phase 3 involves rapid accumulation of synapses. In the macaque primary visual cortex this phase begins two months before birth (about 100 days after conception) and the maximal density of synapses is reached about two months after birth. The most rapid accumulation of synapses is observed around birth (in the macaque, delivery occurs 165 days after conception), when 40 000 new synapses are formed every second in each striate cortex of the macaque (Bourgeois & Rakic, 1993). The time course of this phase has been observed in all the cortical areas investigated in the macaque (Rakic *et al.*, 1986; Zecevic *et al.*, 1989; Zecevic & Rakic, 1991; Bourgeois & Rakic, 1993; Bourgeois *et al.*, 1994; Granger *et al.*, 1995). In the human primary visual cortex, data from Zecevic (1998) suggest that phase 3 starts around mid-gestation (20–24 weeks of gestation). Data from Huttenlocher and Dabholkar (1997) suggest that it ends between 8 and 12 months after birth in the visual cortex and around two to three years in the prefrontal cortex. More precise timescales require studies with greater numbers of samples. In addition, in the human cerebral cortex the most rapid accumulation of synapses is observed around birth. It is fascinating that in every second of the perinatal life of the baby, hundreds of millions of synapses (billions?) are formed in its cerebral cortex. These new synapses are now formed mainly on dendritic spines (Figs. 5.1 and 5.2) of the rapidly growing dendritic arborizations, conjointly with growth of axonal branching. This massive cytoskeletal and membrane biogenesis involves synthesis of vast amounts of proteins and lipids, which continues for several months.

The formation of each synapse is preceded by innumerable transient contacts between thin filopodial extensions from growing axonal and dendritic branches. These filopodial contacts are very short, in the minute range. It has been hypothesized that they allow "prescreening" of synaptic selectivity involving cell surface compatibility between neuronal partners (Lohmann & Bonhoeffer, 2008). When filopodia are cytologically compatible, intracellular calcium-dependent signaling engages the maturational process of these protosynapses (Lohmann & Bonhoeffer, 2008). This involves the time- (in hours) and energy-consuming process of assembling the numerous and diverse scaffolding molecules and pharmacological receptors and channels, forming a morphologically and physiologically mature synapse. Only a small fraction of the filopodial interactions switch to this maturational cascade.

The molecular mechanisms involved in the onset and first steps of synaptogenesis appear to be intrinsic to the cerebral cortex and common to the whole cortical mantle. In 2007, a new genetic picture of synaptogenesis emerged with global analysis of neuronal transcriptomes during development. A coordinated expression of gene clusters, coding for many synaptic proteins, precedes the morphological and electrophysiological differentiation of synapses (Valor *et al.*, 2007). These genes are involved in the prenatal elaboration of synaptoarchitectony (Horton & Hocking, 1996).

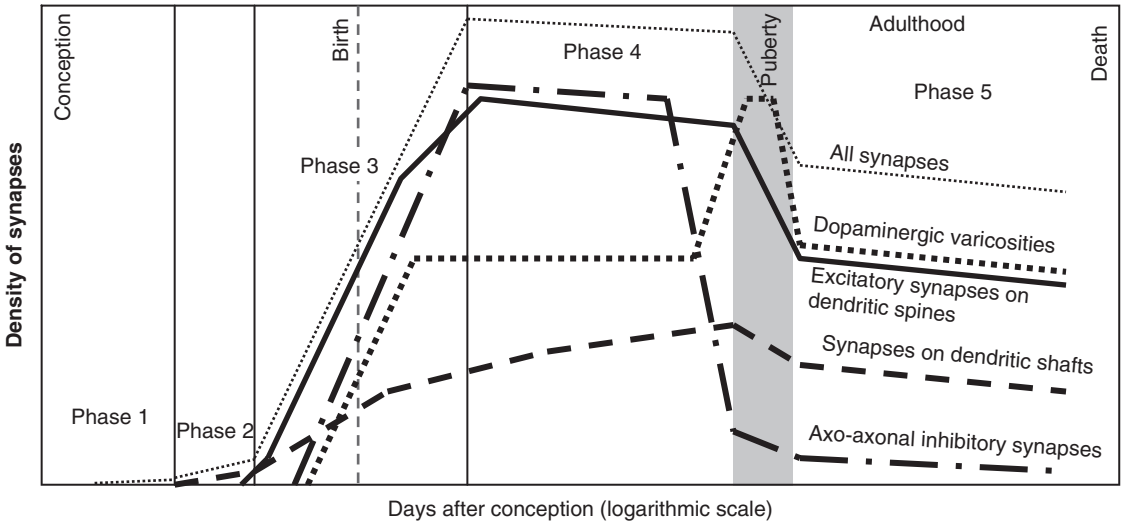

Fig. 5.2 Waves of synaptogenesis during infancy and adolescence. Distinct classes of synapses appear in specific waves of synaptogenesis everywhere in the cerebral cortex of the macaque monkey during normal development. This is illustrated here with densities of diverse classes of synapses presented as a function of days after conception, in the supragranular cortical layer III of the prefrontal cortex of the macaque monkey. Synapses on dendritic spines (solid line) and shafts (long dashed line) present distinct tempos and amplitudes. Inhibitory γ-aminobutyric acid (GABA)ergic cartridge synapses on pyramidal neurons (long-dash/dot line) appear later and are pruned earlier than the excitatory synapses. Dopaminergic synaptic varicosities (thick dotted line) present a peak of density during puberty. Senescence is not represented here. (After Anderson *et al.*, 1995, with permission from Elsevier.)

Synaptogenesis initially proceeds in the absence of patterned stimuli from the external world; that is, it is said to be experience-independent (Fig. 5.1). However, spontaneous neuronal activity is directly involved (Goddard *et al.*, 2007; see also Chapter 10). This is followed by activation of another set of genes, now coding for synaptic proteins sensitive to evoked activity (Valor *et al.*, 2007). They most likely participate in the rapid development of highly specific cortical functions in the neonatal period (Rodman, 1994; Goldberg, 2004) or even before birth (Granier-Deferre *et al.*, 2004).

Phase 4: Synapses "make waves" during infancy and adolescence

During phase 4, "the plateau phase," the mean density of synapses remains at a very high level, 600–900 millions synapses per mm^3 of neuropil, for several years throughout infancy and adolescence, until puberty (which begins in the third year in the macaque). This plateau is observed in the sensory, motor, and association cortices (Rakic *et al.*, 1986; Zecevic *et al.*, 1989; Zecevic & Rakic, 1991; Bourgeois & Rakic, 1993; Bourgeois *et al.*, 1994; Granger *et al.*, 1995). In the human prefrontal cortex the phase 4 "en plateau" lasts for a full decade, until puberty (Huttenlocher & Dabholkar, 1997). In the primary visual cortex, the description of a two- to three-year-long plateau phase is possibly biased by paucity of data during the adolescence period. Several quantitative studies have shown that the curves presented in Fig. 5.1 are "envelope" curves encompassing many waves of synaptogenesis. Distinct waves of synaptogenesis on dendritic spines or shafts (Fig. 5.2) have been identified at the electron microscopic level, in different cortical layers and in diverse cortical areas (Rakic *et al.*, 1986; Zecevic *et al.*, 1989; Zecevic & Rakic, 1991; Bourgeois & Rakic, 1993; Bourgeois *et al.*, 1994; Granger *et al.*, 1995). In Golgi-stained tissues, dendritic spines appear first in sublayer IVCβ then in IVCα primary visual cortex (Lund & Holbach, 1991). In layer III of the prefrontal cortex, distinct waves of synaptogenesis for excitatory dendritic spines, inhibitory cartridge synapses, and modulatory dopaminergic varicosities (Fig. 5.2) have also been observed at the light microscopic level (Anderson *et al.*, 1995).

In layer III of cat primary visual cortex, synapses formed by horizontal connections relocate from proximal to distal positions of dendritic branches (Callaway & Katz, 1990). In the human primary visual cortex, the local vertical synaptic circuits (subserving local visual receptive fields) develop during the last third of gestation, while the horizontal long distance cortico-cortical connections (subserving visual context dependency) develop during the first postnatal year (Burkhalter

et al., 1993). Maturation of cortical inhibitory interneurons follows a slower tempo than the excitatory pyramidal neurons (Murphy *et al.*, 2005). New transgenic methods for tagging neurons will allow extensive simultaneous quantitative analysis of distinct classes of neurons and synapses in vivo and in vitro (Livet *et al.*, 2007). No doubt many more waves of synaptogenesis will be identified. The complex kinetics of synaptogenesis, the presence of multiple waves of synaptogenesis, the modifications in pharmacological receptor compositions or their microphysiological properties (Ben-Ari *et al.*, 2007) underline the fact that the synaptoarchitectonic organization of the cerebral cortex significantly varies throughout normal development and maturation. Some of these modifications may even interact with maternal hormones during delivery (Tyzio *et al.*, 2006). The consequences of these normal developmental reorganizations for the learning and representational abilities of the cerebral cortex have not yet been evaluated. The last phases of synaptogenesis are just outlined below.

The synaptic catastrophe at puberty

In all cases, the density of synapses markedly decreases in the pubertal period (Rakic *et al.*, 1986; Zecevic *et al.*, 1989; Zecevic & Rakic, 1991; Bourgeois & Rakic, 1993; Bourgeois *et al.*, 1994; Granger *et al.*, 1995). This reduction, by at least 40% (Figs. 5.1, 5.2, and 5.3),

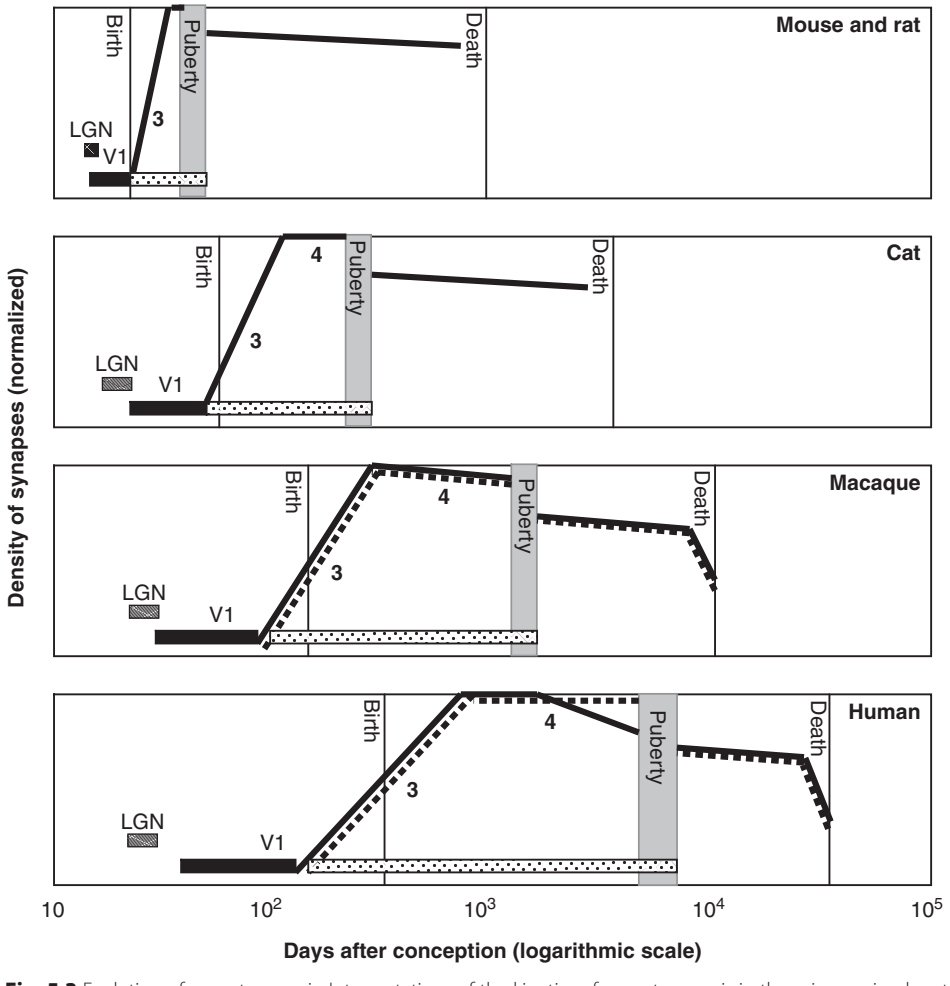

Fig. 5.3 Evolution of synaptogenesis. Interpretations of the kinetics of synaptogenesis in the primary visual cortex (black line) and prefrontal cortex (dashed line) are schematized here for a few mammalian species. The density of synaptic contacts (normalized to maximal value, on cartesian *y* axis) is presented as a function of the number of days after conception (logarithmic *x* axis). To simplify the illustration, only phases 3, 4, and 5 are indicated. The time periods of neurogenesis in the lateral geniculate nucleus (LGN; striped horizontal bars) and primary visual cortex (V1; solid horizontal bars) are represented. The dotted horizontal bars represent the timespan of all the critical periods together. The representation of the onset of some critical periods before birth is putative and requires further investigation.

most likely results from a true loss of synapses as observed in the macaque. The physiological causes of this synaptic pruning remain unidentified.

Phase 5: Discrete synaptogenesis in adult life

Phase 5 consists of a very slow and steady decline in density of synapses (Figs. 5.1 and 5.3) from the end of puberty throughout adulthood (Rakic *et al.*, 1986; Zecevic *et al.*, 1989; Zecevic & Rakic, 1991; Bourgeois & Rakic, 1993; Bourgeois *et al.*, 1994; Granger *et al.*, 1995). In the adult cerebral cortex, synaptogenesis is still an experience-dependent process but quite restricted and local. The geometries of axonal and dendritic arborizations are not reshaped by individual experience. Finally, at the end of life, a marked loss of synapses is observed during senescence.

These five distinct phases of synaptogenesis have been identified using quantitative electron microscopy, and fit well with observations from immunocytological investigations (Anderson *et al.*, 1995) or imaging of the macaque (Jacobs *et al.*, 1995) and human (Chugani *et al.*, 1987) cortices.

Robustness and sensitivity of the early phases of synaptogenesis

In this section we discuss the core problem of synaptogenesis. The neurons are genetically programmed to produce a given amount of synapses per cubic millimeter of the cortex, regardless of the histological and physiological conditions. In the same time, the synaptoarchitectonic organization becomes highly sensitive to environmental conditions. The early phases of massive production of synapses are quite robust. Surgical alteration of the cortical circuits during early fetal life does not alter the global production of synapses during phase 3 in the cortical tissue, but it modifies permanently the proportions of synapses on spines and on shafts (Bourgeois & Rakic, 1996). Experimental prematurity does not alter the global kinetics of synaptogenesis in the primary visual cortex during phase 3, but it does alter transiently the synaptoarchitectony in the thalamoceptive cortical layers (Bourgeois *et al.*, 1989). Clear alterations are observed in the sizes and proportions of synapses located on dendritic spines or shafts, as well as the ratios of asymmetrical to symmetrical synapses, at the early stages of experimental prematurity. The alterations disappear according to the time of development. Many observations on mice bearing a variety of null mutations report an apparently normal density of synapses, but few describe the synaptoarchitectony quantitatively. In knockout mice lacking the β2 subunit of the high-a nity neuronal nicotinic cholinergic receptors the mean density of synapses remains unchanged in the adult cerebral cortex (Bourgeois, 2009, unpublished data). In contrast, the extension of the basolateral dendritic fields, the dendritic arbor geometries, and the densities of dendritic spines along dendritic branches, are markedly modified (Ballesteros-Yáñez *et al.*, manuscript in preparation). Several altered conditions of neonates have notable effects on the cerebral cortex. Maternal or paternal deprivation during the first three postnatal weeks results in fewer synapses on dendritic shafts of pyramidal neurons in layer II of the cingulate cortex of adult rodents (Helmeke *et al.*, 2001). Diverse levels of maternal care have diverse physiological consequences (Sullivan *et al.*, 2006; Champagne *et al.*, 2008). These very early alterations of the neonatal environment affect developing synaptic networks, resulting in discrete synaptoarchitectonic modifications that last through adulthood and can be associated with functional alterations such as heightened emotionality. Interestingly, the birth of a baby also affects synaptoarchitectony in adults: fatherhood triggers synaptogenesis in the prefrontal cortex of prosimian fathers (Kozorovitskiy *et al.*, 2006). So how do robustness and sensitivity coexist during synaptogenesis?

Effects of genes and environment: an enduring story of synaptic plasticity

Genes and the environment are no longer considered to be in opposition. They cooperate quite dynamically, with synapses as their "go-between." The genes construct the synaptoarchitectony in the cerebral cortex of each human being within a window of natural variability (Larsen, 2005) before it is refined by interactions with the environment. This is the theoretical framework behind selective stabilization of developing synapses (Changeux & Danchin, 1976). The resulting adult cortical synaptoarchitectony generates behaviors that are targets for natural selection (Krubitzer, 2007). The synaptic plasticity allows the synaptoarchitectony to adapt physiologically and morphologically to gene expression patterns and to patterns of action potentials propagating in the networks. Synaptic plasticity is mediated by microphysiological regulation in each synapse of the assortment of pharmacological

receptors, enzymes, cell adhesion molecules, cytoskeletal proteins – their local synthesis, tra c, and lateral diffusion (Levi *et al*., 2008), chemical modifications (phosphorylation/dephosphorylation), assembly–deassembly of molecular scaffoldings, etc. At the morphological level, synaptic plasticity is mediated by reorganization of the synaptoarchitectony via production/retraction of axonal and dendritic branches, formation/elimination of synapses, and ultrastructural remodeling of dendritic spines. These multiple variations in the e cacy of synaptic transmission constitute the currently dominant paradigm for the morpho-functional bases of learning and memory in the cerebral cortex. The ongoing discussions are about the prevalence of the presynaptic or postsynaptic domains and on the nature of discrete functional states of synapses (Bourgeois, 2005). In addition, there is a synaptic metaplasticity, in which the e ciency of synaptic transmission is also modulated by competition and/or cooperation between neighboring synapses along dendritic branches (Rabinowitch & Segev, 2008). There is not enough room here to describe the multiple contributions of the diverse neuromodulations to synaptic plasticity (Fuxe *et al*., 2007).

Critical periods in synaptogenesis

Earlier in the chapter, we touched on the paradoxical coexistence of robustness of synaptogenesis along with high sensitivity of synaptoarchitectony to the environment. How can these characteristics cooperate to build the highly specific synaptic networks in the cerebral cortex? Critical periods in synaptogenesis might be the key to this cooperation, by adding enhanced synaptic plasticity to the robust triggers of synaptogenesis (Fig. 5.1). During these so-called critical periods the amplitude of sensitivity of synaptic plasticity to the evoked neuronal activities representing the environment becomes maximal (Hooks & Chen, 2007; Morishita & Hensch, 2008). The refinement of the synaptoarchitectony becomes experience-expectant (Fig. 5.1). The presence of normally patterned action potentials propagating in synaptic networks is required for the refinement of synaptoarchitectony and optimal cortical functions.

Mechanisms opening the critical periods

Multiple genetic and epigenetic mechanisms control the onset, duration, and closing of the critical periods (Berardi *et al*., 2000; Bartoletti *et al*., 2004; Hooks & Chen, 2007; Morishita & Hensch, 2008). They have been mostly investigated in the primary visual cortex. The release of trophic factors such as brain-derived neuronal factor (BDNF) by postsynaptic neurons is proportional to the level of presynaptic evoked activity. The presynaptic uptake of BDNF increases the number and stability of synapses. This growth factor relates neuronal activity to neuronal axonal and dendritic growths and this effect is maximal during critical periods of the formation of cortical ocular dominance columns. The diverse synaptic functional states are in league with networks of genes via multiple cascades of intracellular signaling pathways. Presynaptic binding of BDNF controls gene transcription via transcription factors such as cAMP-response element binding (CREB) proteins. During the critical period, a monocular deprivation activates gene expression under control of CRE. Evoked activity activates gene*Cpg15* (candidate plasticity gene 15) and the expression of protein cpg15 in the growing axonal branches. This activation is maximal during the critical period of formation of the ocular dominance columns in the visual cortex. Numerous activity-dependent posttranscriptional modifications of messenger RNA are also involved. Synaptic inhibition mediated by -aminobutyric acid (GABA) participates in the opening and closing of critical periods. The visual cortex of mice bearing the null mutation for glutamic acid decarboxylase (GAD65), an enzyme involved in the synthesis of GABA, is insensitive to monocular deprivations during the corresponding critical period (Spolidoro *et al*., 2009). Evoked activity in the visual system increases expression of major histocompatibility complex class I (MHC I) ligands and their receptors (Goddard *et al*., 2007). This sensitivity is maximal during the critical period of development of synaptic circuits specifically expressing these transmembrane proteins. In mice bearing null mutations for these proteins, the axonal pruning is defective. Synapses are formed anyway (robustness of synaptogenesis) but synaptic microphysiology (potentiations and depressions) and synaptoarchitectony are altered (sensitivity of synaptogenesis).

Mechanisms closing the critical periods

In excitatory glutamatergic synapses, the substitution of subunit *N*-methyl-D-aspartate (NMDA)-R_{2B} (slow synaptic currents) with subunit NMDA-R_{2A} (fast synaptic currents) coincides with the closing of critical periods for sensory deprivations in somatosensory,

auditory, and visual cortical areas. The accumulation of polysialic acid, a transmembrane glycoconjugate, on the neuronal cell surface culminates at the end of critical periods and results in marked reduction of the possibilities of reorganizing the synaptoarchitectony (Rutishauser, 2008; Spolidoro *et al.*, 2009). Myelination of axons also participates in the waning of synaptic plasticity (McGee *et al.*, 2005).

Multiple critical periods follow one another from the end of gestation to the end of puberty (phases 3 and 4 in Fig. 5.1). Their time windows differ according to the mammalian species (Fig. 5.4), the diverse sensory, motor, or cognitive modalities, and the distinct brain compartments and cortical layers serving each of these cortical functions. Critical periods occur first in the visual thalamus, then in granular layer IV of the visual cortex, then in the supragranular layer III, etc. Extensions of critical periods for cognitive functions, or social behaviors, have not been addressed yet.

Evolution of synaptogenesis and the critical periods

Evolution of synaptogenesis

The observations of synapses in all metazoans, except the porifera, indicate that they are ancient, specialized subcellular structures. The study of evolution of synaptogenesis did not start until we exploited the available kinetics of synaptogenesis described mostly in primary visual cortices of several mammalian species (Bourgeois, 1997). Throughout evolution, the duration of all phases of synaptogenesis increases along with the size of the cerebral cortex.

The time window of massive production and elimination of synapses in the cerebral cortex extends from the onset of phase 3 prenatally until the end of phase 4 of synaptogenesis post puberty. This time window is about 200 times longer in the human cortex than in the murine cortex (Fig. 5.4). However, this extension in time does not yield a 200-fold higher density of synapses per cubic millimeter of human cerebral cortex as compared with the mouse. Remember, it takes only about 30–120 minutes to make a synapse, which fills only an infinitesimal fraction of the volume in that cubic millimeter of cortex (Lohmann & Bonhoeffer, 2008). The genes that govern this extension in time have not been identified yet. In the heterochronic epigenesis hypothesis (HEH), we proposed that this extension in time of synaptogenesis might also result from the histological and physiological heterogeneities of the cerebral cortex, which increase significantly through mammalian evolution (Bourgeois, 1997). The physicist John Archibald Wheeler said that "Time is Nature's way of keeping everything from happening all at once." This succinctly illustrates that time is a crucial parameter for the evolution of synaptogenesis in the cerebral cortex. To the window of wiring variability controlled by genes, this HEH also adds time as a generator of epigenesis through recursive effects at synapses. These recursive mechanisms allow themselves more cytological interactions and more physiological signals to be extracted or/and integrated between larger, heterogeneous groups of neurons, in order to refine huge numbers of synaptoarchitectonic fields. This is even more relevant for the cognitive fields. The prefrontal cortical areas, serving the most elaborated cognitive functions, receive the most diversified axonal inputs from the whole cortical mantle (Fuster, 2001). These prefrontal fields have to wire together highly cytologically and functionally heterogeneous neurons (Wang *et al.*, 2006). The durations of their critical periods, although not yet explored, might be extensive. So, please, give infants (and adults) the time they need!

We do not yet know the molecular code specifying these synaptic networks. The 25 000–30 000 human genes are said to be insu cient for determining each of the estimated 10^{14}–10^{15} synapses formed in the human cerebral cortex (Braitenberg & Schüz, 1998). Kerszberg (2003) calculated that "each synapse in the brain could be specified at the cost of roughly 50 molecular labels used combinatorially." According to his argument, it is even surprising that there is still some synaptic plasticity in the cerebral cortex. But synaptic plasticity does exist, even in the adult cortex. Cell adhesion molecules involved in the recognition and sticking of the presynaptic and postsynaptic cytoplasmic membranes during synaptogenesis are good candidates for the control of this complex process of synaptic specification (Shapiro *et al.*, 2007; Takeichi, 2007). The sequencing of the human genome has revealed that the families of cell adhesion molecules are significantly expended in the human species (Venter *et al.*, 2001), along with more alternative splicing generating huge diversity of proteins (Lander *et al.*, 2001). The low a nities of these cell adhesion molecules (K_D in the micromolar range) would allow turnover and reorganization of synaptic scaffolding molecules. I hypothesize that this would provide the

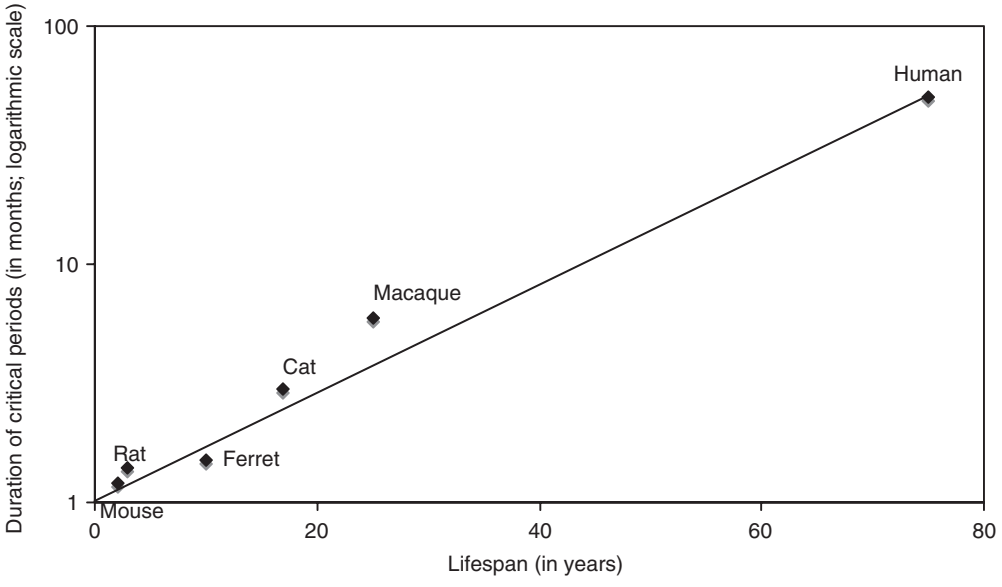

Fig. 5.4 Evolution of critical periods. The figure shows the duration of the critical period for primary visual cortex sensitivity to monocular deprivation in several mammalian species. The duration of this critical period ranges from about a month in the mouse to several decades in the human cerebral cortex. (After Berardi *et al.*, 2000, with permission from Elsevier.)

HEH with flexible molecular coding rules for assembling and diassembling synapses during the distinct phases of synaptogenesis.

Evolution of the critical periods of synaptic plasticity

The duration of the critical periods increases markedly throughout mammalian evolution. The critical periods last for a few weeks in the murine cerebral cortex and about two decades in the human cerebral cortex. According to our present knowledge, this prolongation of the timespan of critical periods reaches its maximal value in the human cerebral cortex. This is illustrated in Fig. 5.4, with the increased duration of critical periods for the sensitivity of the visual cortex to monocular deprivation in several mammalian species (Berardi *et al.*, 2000).

This temporal extension allows maximal amplitude and duration for synaptic plasticity and numerous local refinements of synaptic circuits. It is correlated with the long and complex development of human sensory and motor skills, the acquisition of complex social rules in human and nonhuman primates groups, and the immense cultural corpus continuously produced in our human societies. This extension of the period of maximal synaptic plasticity for decades and the epigenetic opening of synaptogenesis to environment are maximal in the human cerebral cortex. They are the sources of the exceptional cognitive adaptability of our species and possibly also a major source of its fragility.

Synaptic pathologies

It is tempting to relate the diverse onsets of several major neurodevelopmental syndromes defined by deficits in social reciprocity, communication, and unusual behaviors to the different phases of synaptogenesis described above. Fragile X and Down syndromes become evident during the perinatal period (early phase 3). Autistic syndromes become manifest within the first two to three postnatal years (second part of phase 3), within two to ten years (phase 4 "the plateau") for the Rett syndrome, and around the end of puberty (end of phase 4) for schizophrenic syndromes. Genetic linkage analyses of these syndromes indicates an association with an increasing number of mutations of synaptic proteins, among other proteins. Mutations of synapsin are linked to schizophrenia (Frankle *et al.*, 2003). It is hypothesized that these mutations result in disconnections of higher-order association areas from the frontal lobe during development (Geschwind & Levitt, 2007; Sutcliffe, 2008). Mutations of synaptic cell adhesion molecules neuroligins and neurexins, scaffolding protein shank 3, and mutations in the melatonin synthesis pathway are

linked to autistic syndromes (Bourgeron, 2007, 2009). In autisms, alterations of synaptic plasticity might be related to circadian rhythms disorders. The multiplicity of factors involved in the functional and morphological alterations of synaptic circuits hampers a quick appreciation of the etiology of these syndromes.

First, the causal chain between the mutated genes, the alterations of synaptic circuits, the symptoms, and the environments is not known. Whether these syndromes result from a fault in normal early development of synaptic circuits or from a deficit in synaptic plasticity involved in their late refinement remains an open question. The assortment of genetic alterations and environments is distinct for each patient. Defective genes might result in defective synaptogenesis, producing altered behaviors. The social environments confronting these altered behaviors are themselves modified. Effects of modified environments on genetic expression patterns might themselves be altered. In this causal loop, it is still di cult to assess the contribution of each parameter to the defective cortical synaptoarchitectony.

Second, tentative animal models have been developed to address these questions. They are used in experiments to identify endophenotypes and potential therapeutic strategies. However, what is the validity of animal models for human psychiatric pathologies (Abbott, 2007; Low & Hardy, 2007)? Does the transfer of mutated human genes to mice transfer the human pathologies to the mice (Jamain *et al.*, 2008)? The human brain is not simply a big mouse brain and the list of its singular features is currently increasing. The overall size of the human cerebral cortex and its pattern of convolutions are obvious. It has the largest number of cortical areas, with some unique ones (Bourgeois, 1997). It has the longest kinetics of synaptogenesis for establishment and refinement of the synaptoarchitectony (Bourgeois, 1997) and the longest duration for the critical periods of functional maturation (Fig. 5.4, after Berardi *et al.*, 2000). At the cellular level it has the largest percentage and diversity of interneurons (Ascoli *et al.*, 2008), the largest size of pyramidal neurons, the highest density of dendritic spines on dendritic branches, and the largest size of dendritic spines (Benavides-Piccione *et al.*, 2002). New types of fusiform neuron have been identified in layer V of the anterior cingulate cortex (Nimchinsky *et al.*, 1999). The human brain permanently displays the largest number of active genes, and the largest number of mRNA transcripts. Genes specific to the human brain are being identified (Hayakawa *et al.*, 2005). At the physiological level, the human brain has the highest neurophysiological activity, the richest proteome (Enard *et al.*, 2002), and the highest metabolic activity (Cáceres *et al.*, 2003).

Epigenetic manipulations of synaptic circuits

Neurobiologists are currently learning how to control the opening and closing of critical periods of synaptogenesis using animal experimental models. Diverse genetic, pharmacological, biochemical, and environmental procedures are being exploited. Modulating the balance between inhibition and excitation is crucial. Diazepam, an agonist for the GABAergic receptor GABA-R_A, increases synaptic plasticity, allowing advancement of the onset of critical periods, prolongation of their duration, and even reinstating them in the adult cortex (Hooks & Chen, 2007; Morishita & Hensch, 2008; Spolidoro *et al.*, 2009). Fluoxetine, an inhibitor of serotonin uptake, modifies the rates of neurogenesis and synaptogenesis in adult circuits (Maya Vetencourt *et al.*, 2008). The depolymerization of the extracellular matrix by enzymatic treatment, associated with a supply of nerve growth factors, restores synaptic plasticity (Bradbury *et al.*, 2002; Pizzorusso *et al.*, 2002; Rutishauser, 2008). Enriched environments do restore plasticity in adult synaptic networks in a modality-specific way. Placing rats in a large space to explore results in increased density of synapses in their primary motor cortex (Kleim *et al.*, 1996). An environment enriched with odors stimulates neurogenesis and synaptogenesis in the olfactory bulb of adult mice (Alonso *et al.*, 2008). Restoration of synaptic plasticity may also exploit functional interplays between distinct modalities. Rearing of animals in the dark slows down the functional and morphological maturation of synaptic circuits. In contrast, when these animals are reared in a dark but enriched environment, one observes a restoration of synaptic plasticity and maturation in the deprived visual sensory modality (Cotman & Bertchtold, 2002; Bartoletti *et al.*, 2004). The interpretation of this effect is not well documented yet. An enriched environment, activating other sensory, motor, and cognitive modalities, might act via inter-modal connections and/or neuromodulatory networks, and/or a reduction of cortical inhibition (Sale *et al.*, 2007). During the critical periods it is also possible to slow down the anatomical

and functional maturation of the synaptic circuits by placing individuals in an impoverished environment. Rearing pups in stroboscopic light extends the duration of the critical period of development of the primary visual cortex (Fagiolini *et al.*, 1994). Development in the presence of continuous white noise prolongs the duration of the developmental critical period for the primary auditory cortex (Chang & Merzenich, 2003; Wang, 2004). A reasoned manipulation of these epigenetic properties might also be exploited to repair pathological synaptogenesis. One may anticipate treatments combining together several of the methods discussed above to reinstate synaptic plasticity in defective developmental processes to reorient them properly.

Conclusion

If one is challenged to sum up in a single sentence the whole story of synaptogenesis in the human cerebral cortex, the exercise might look like this: "For about 300 millions years, the evolution of cerebral cortex has involved selecting networks of genes, controlling the kinetics of synaptogenesis, and allowing the environment to refine the synapto-architectony within a window of variability." Through evolution of the mammalian cerebral cortex, the time required by networks of genes to trigger the formation of the very first synapses in the fetal cortex increases moderately compared with the marked increase in duration of gestation. As a result, the onset of synaptogenesis, which is a perinatal event in rodents, happens very early in human gestation. In the last months of gestation, networks of genes trigger an "explosion" in the production of synapses, extending well into the third postnatal year. This coincides with the development of sensorimotor skills and language. The phase with a high density of synapses, "the plateau," is maintained for a decade, during the time when the individual learns social and cultural rules. Puberty – the period of sexual transformation along with intellectual individualization, emancipation from the immediate parental environment, and access to social autonomy – is accompanied by a marked reduction in the number of synapses. The steady adult synaptic numbers overlap with the protracted time for human cultural refinement, creativity, production of knowledge, and transmission of life and values. Multiple forms of synaptic plasticity provide the physical support necessary for learning and memory through all these phases of life. Evolution has resulted in selected transient critical periods of synaptogenesis when cooperation between genes, synaptic plasticity, and the environment becomes maximal.

Groups of neurons are highly heterogeneous, differing in their gene expression patterns, intracellular communication pathways, their morphological and biochemical phenotypes, pharmaco-receptivity and functional states, etc. Thus, synaptogenesis is only one aspect of cerebral cortex development and maturation. However, alterations of synaptoarchitectony are always associated with cognitive dysfunction. Multiple mutations of genes coding for synaptic proteins are currently identified as being linked to diverse neuropsychiatric syndromes. In the present working hypothesis, diverse assortments of mutated genes control the formation of altered synaptoarchitectonies, producing altered behaviors and inducing modified environments, which themselves differentially activate the genes networks. Such causal loops are being investigated. Neurobiologists are presently learning how to manipulate the synaptic plasticity and critical periods of synaptogenesis during development and in the adult cerebral cortex. No doubt combinations of these epigenetic manipulations will be exploited to repair the synaptic alterations associated with mutations of synaptic proteins with the intention of reinstating normal maturation of synaptoarchitectony. They open fascinating therapeutic opportunities for human neuro- and psychopathologies. They will also touch the core of the individual human mental nature and freedom. As usual, progress has two faces. As usual also, fears of scientists are anticipated by poets, as when René Char wrote: "Le cerveau, plein à craquer de machines, pourra-t-il encore garantir l'existence du mince ruisselet de rêve et d'évasion ?" (in *Fureur et Mystère*, 1948). This might be translated as: "Full to bursting with machines, will the brain still be able to safeguard the existence of the tiny rivulet of dream and escape?".

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Chapter 6

Neurotrophic factors in brain development

Thomas Ringstedt

Neurotrophic factors are target-derived survival factors

During nervous system development, neurons are generated in numbers exceeding those found in adults. The surplus neurons are eliminated by programmed cell death. In higher organisms this process is influenced by factors outside the cells themselves, called neurotrophic factors. The discovery of neurotrophic factors goes back to the 1950s, when Rita Levi-Montalcini was investigating the relationship between the nervous system and its peripheral targets. She and her collaborators found that on removal of an organ from a chick embryo, its innervating ganglion neurons were lost as well. Conversely, the addition of an extra target resulted in excessive neuronal survival. She postulated that a soluble factor produced by the target promoted neuronal survival. Eventually this factor was isolated and named nerve growth factor (NGF; Cohen & Levi-Montalcini, 1957). A related protein was purified from pig brain in 1982, and was named brain-derived neurotrophic factor (BDNF; Barde *et al.*, 1982). With the discovery of BDNF, a family of neurotrophic factors was established: the NGF family of neurotrophic factors, or the neurotrophins. Since then the mammalian branch of the family has been gifted with two additional members, neurotrophin-3 (NT-3) (Hohn *et al.*, 1990) and NT-4 (Hallbook *et al.*, 1991). To date several proteins with neurotrophic action have been described. Apart from the neurotrophins, members of the glial cell line-derived (GDNF) factor family are the most important.

Neurotrophic factors and their receptors are expressed during development and in adulthood. The original concept as target-derived survival factors for innervating neurons still holds. In higher organisms, competition for neurotrophic factors during the period of naturally occurring cell death weeds out ill-positioned neurons. Since neurons are produced in excess, this mechanism acts to fine-tune connections between neurons and their targets, and balance their number with the size of the target tissue. Neurotrophin homologs are absent in invertebrates, and it is therefore possible that the plasticity inferred by an extrinsic regulation of neuronal survival (as opposed to cell-intrinsic regulation) has co-evolved with higher neuronal complexity. In addition to their role as target-derived survival factors, several other functions are now attributed to the neurotrophins. This is particularly obvious in the brain, where these other functions probably predominate. What follows is a general introduction to the neurotrophins, and a summary of their functions during central nervous system (CNS) development.

The neurotrophins

Structure

The neurotrophins, NGF, BDNF, NT-3, and NT-4, are di usible factors active in homodimers. Heterodimers between di erent members of the family can be assembled in vitro, but there are no indications that heterodimers are operational in the nervous system. The neurotrophin monomers are first produced as proneurotrophins. The prodomain is subsequently removed by proteolysis to form a mature protein. The proform can form homodimers and has a biological function in part distinct from that of the mature protein (Lee *et al.*, 2001; Teng *et al.*, 2005). The mature monomer is composed of about 120 amino acids, half of which are identical within the family. The monomer can be further subdivided into conserved and variable regions. Most of the variable regions have been implicated in receptor binding (for a review, see Ibanez, 1998).

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Receptors and signaling

The neurotrophins signal through two classes of receptors: the p75 neurotrophin receptor (p75NTR) and the tropomyosin receptor kinase (Trk) receptors. Although discovered first, the biological roles and signaling pathways of the p75NTR receptor were for a long time less well characterized than those of the Trk receptors.

There are three Trk receptors in mammals: TrkA, TrkB, and TrkC. They are members of the receptor tyrosine kinase family, and exist as transmembrane receptors with a tyrosine kinase activity in the intracellular region. Alternative splicing creates various subforms of the Trk receptors, some of which are "truncated," lacking a considerable part of their cytoplasmic region, including the tyrosine kinase domain. These subforms have altered ligand preferences, and restricted signaling capacity (in the truncated forms). The di erent neurotrophins bind to and activate distinct Trk receptors according to the following scheme: NGF binds to TrkA, BDNF and NT-4 are both ligands of TrkB, whereas NT-3 binds TrkC. NT-3 can also bind to and activate the other Trk receptors, but with less e ciency. The neurotrophin dimer binds two Trk receptors, bringing them together and thereby enabling transphosphorylation of the kinases in their cytoplasmic domains. This activates several signaling pathways. These include Ca^{2+} release via phospholipase C (PLC)γ1 and inositol 1,4,5-triphosphate (IP3), promotion of survival via phosphoinositide 3 kinase (PI3k) and Akt, and induction of di erentiation via Ras/mitogen-activated protein kinase (MAPK) (for reviews, see Ibanez, 1998; Reichardt, 2006). Although BDNF and NT-4 both act via the TrkB receptor, downstream signaling can still proceed di erently (Minichiello *et al.*, 1998). Nerve cells can be very elongated structures. Therefore, for a target-derived neurotrophin ligand to a ect gene transcription (e.g., when promoting survival), it first has to undergo retrograde transport to the cell nucleus. The receptor ligand complex is in some instances internalized and transported via endosomes to the cell soma. Neurotrophins can also act locally, without retrograde transport. An interesting example is given by the sympathetic ganglion neurons. During embryonic development they are exposed to NGF and NT-3, although they only express TrkA receptors. In this system, both ligands act via the TrkA receptor, but with di erent outcomes. NGF and NT-3 act locally to promote sympathetic axon growth, but only NGF can signal survival because only the NGF–TrkA complex is capable of undergoing retrograde transport (Kuruvilla *et al.*, 2004) (Fig. 6.1).

The p75NTR lacks an intracellular tyrosine kinase or other catalytic motif. It is part of the tumor necrosis receptor superfamily, and, like the other members of this family, its cytoplasmic domain includes a "death" domain. p75NTR binds all neurotrophins with equal a nity. Ligand binding can activate several signaling pathways, mediated by various adapter proteins binding to the p75NTR. These can a ect growth cone motility via RhoA, signal survival via nuclear factor (NF)-κB, or perhaps most importantly promote apoptosis via the Jun kinase-signaling cascade. Interestingly, proneurotrophins are able to bind p75NTR (but not Trks), and therefore tend to signal apoptosis rather than survival. The p75NTR also interacts with Trk receptors. Together with TrkA it can form a high-a nity binding site for NGF, enhance Trk signaling, and promote endocytosis and retrograde transport of Trk–neurotrophin complexes (for reviews, see Kaplan & Miller, 1997; Reichardt, 2006).

Neurotrophin and neurotrophin receptor expression

Neurotrophins are expressed in the peripheral target tissues during the period of naturally occurring cell death, but are also found within the peripheral nervous system (PNS) ganglia themselves. Somewhat surprisingly, the Trk receptors are expressed earlier during development than their ligands. In mouse, the earliest detected receptor mRNA encodes TrkC, and it is reported to be expressed in the neuroectoderm during late gastrulation, at embryonic day 7.5 (E7.5). The p75NTR is coexpressed in the same populations as Trk receptors.

Within the ganglia, distinct cell populations expressing di erent Trk receptors are present, and they respond to matching survival signals from their respective target tissues. The dorsal root ganglia, for example, contain TrkC-expressing proprioceptive neurons that respond to NT-3 produced by muscles and the spinal cord motor neurons, TrkA-expressing nociceptive neurons that respond to NGF produced in the skin, and other populations of sensory neurons that respond to BDNF or NT-4 (for a review, see Bibel & Barde, 2000).

In the rodent CNS, mRNAs encoding TrkB and TrkC are expressed throughout embryonic development, while expression of TrkA and p75NTR is more spatially restricted and does not begin until shortly before birth

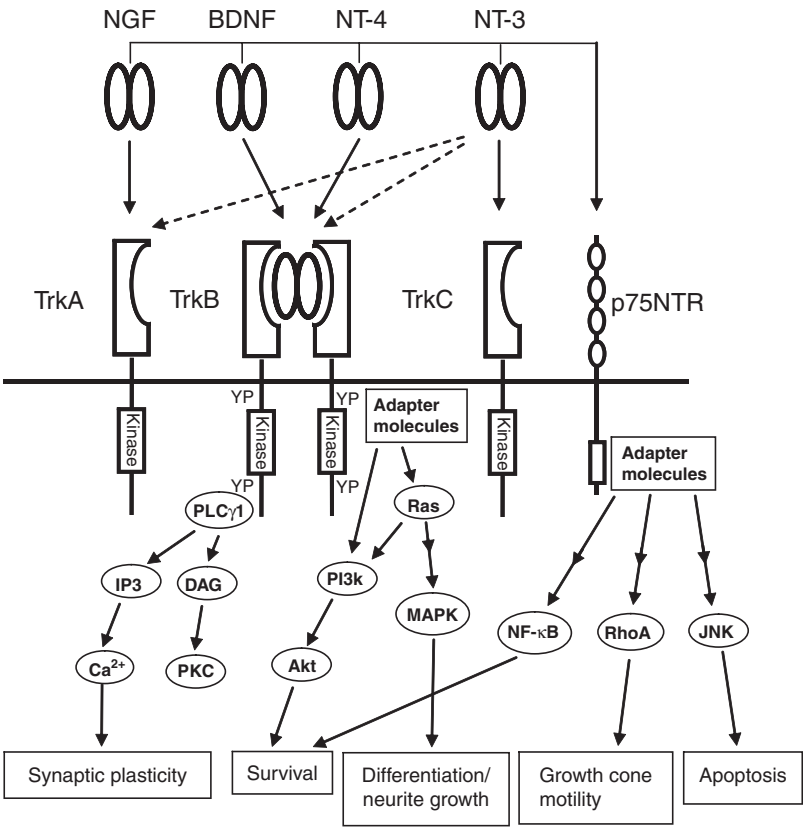

Fig. 6.1 The neurotrophins, their receptors and signaling pathways. The neurotrophins bind to the tropomyosin receptor kinase (Trk) receptors and the p75 neurotrophin receptor (p75NTR). Continuous-line arrows indicate preferred neurotrophin–receptor interactions, whereas the dotted arrows indicate alternative interactions. A neurotrophin dimer binds two Trk receptors, thus bringing them together so that tyrosine transphosphorylation can occur. Intracellular signal transduction then proceeds via different intracellular pathways. Several aspects of cell life are regulated, either directly or indirectly by regulation of gene transcription. All neurotrophins bind to the p75NTR with equal affinity. Although its intracellular domain lacks kinase activity, it can still bind and signal via adapter molecules. The pathways and outcomes are simplified. Double arrows indicate intermediate steps that are left out. NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; PLC γ1, phospholipase C; IP3, inositol 1,4,5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C; PI3k, phosphoinositide 3 kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; JNK, c-Jun-N-terminal kinase.

(Lu *et al.*, 1989; Ernfors *et al.*, 1992; Merlio *et al.*, 1992). NT-3 is the earliest expressed neurotrophin in the mouse brain (Farinas *et al.*, 1996). BDNF expression in the rat neocortex is low before birth, but then rapidly increases, peaking around three weeks after birth (Sugiyama *et al.*, 2003). NGF expression is more restricted to certain cell populations, and the levels in the neocortex and hippocampus are very low prenatally, but then rapidly increase to peak three weeks postnatally (Large *et al.*, 1986). Neurotrophins are also expressed in the adult brain, where they influence maintenance and plasticity. BDNF is widely expressed during postnatal development and adulthood, indicative of its important role in brain maturation and plasticity.

Neuronal survival is promoted by neurotrophins during PNS development

The concept of neurotrophic action is well established in the PNS. As described in the previous section, the neurotrophic factors are expressed in several peripheral target areas, for example skin, while their receptors are found in the innervating ganglia. When the growing neuronal processes reach their targets, they compete for a specific growth factor, expressed by the target in limiting amounts. Excessive and incorrectly projecting neurons are eliminated by programmed cell death, thus adjusting the number of neurons to the size of the target they innervate. By this mechanism, a correct wiring of the nervous system is ensured. In addition, it is possible that the excessive amount of neurons generated and subsequently eliminated is a necessary step in the pattern formation of the tissue in question.

The discovery that neurotrophins are expressed not only by target tissues but also by the neurons themselves and their precursors, cells that also express neurotrophin receptors, led to the concept of local action, i.e., that neurotrophins act in a paracrine or autocrine fashion. This can be exemplified by the role of NT-3 in the developing trigeminal and dorsal root ganglia. If the normal NT-3 production in these areas is knocked out, cell death will occur before target innervation. Furthermore, NT-3 does not act on

mature neurons in this system, but on precursor cells (Farinas *et al.*, 1996). The survival-promoting role of neurotrophins is not limited to neurons or their precursors. An interesting example is given by a recent study demonstrating that in vitro survival of human embryonic stem cells is promoted by BDNF, NT-3, and NT-4 (Pyle *et al.*, 2006).

Neurotrophins as survival factors in the CNS

The essential function of the neurotrophins as survival factors in the PNS has often been extrapolated to the CNS. The expression pattern of neurotrophins and their receptors is compatible with a function as target-derived survival factors and, at least in the case of BDNF and NT-3, also with local action. The concept of neurotrophins as survival promoting in the CNS was supported by early studies on cultured cells. Survival of cultured cholinergic cells from basal forebrain is promoted by NGF and BDNF (Alderson *et al.*, 1990), while survival of cultured motor neurons is promoted by BDNF and NT-3 (Henderson *et al.*, 1993). NT-4 promotes survival of striatal neurons in organotypic slice cultures (Ardelt *et al.*, 1994). The in vitro findings were supported by studies of animal models where neural pathways within the brain were transected. In adult rats, NGF and BDNF prevent degeneration of cholinergic cells of the basal forebrain after transection of the septo-hippocampal pathways (Knusel *et al.*, 1992). BDNF also acts on developing motor neurons after sciatic nerve transections during the prenatal period (Yan *et al.*, 1992). NT-3 rescues norepinephrinergic cells from the locus ceruleus of adult rats from degeneration after chemically induced lesions (Arenas & Persson, 1994). More relevant for explaining CNS development, administration of BDNF to chick embryos prevents the extensive loss of motor neurons that normally occurs during the period of naturally occurring cell death (Oppenheim *et al.*, 1992).

Investigators were given a new powerful tool with the experimental method of targeted gene deletions in mice. Since in vitro and transection data (described above) indicated that neurotrophins can act as (target-derived) survival factors in the CNS, severe neuronal losses were expected in the brains of neurotrophin- and Trk-receptor-deficient (knockout) mice. The PNS of mutant mice displayed neuronal losses in accordance with earlier data obtained by addition of neurotrophins, or the use of blocking antibodies. Surprisingly, this turned out not to be true for the CNS. Despite the abundant and regulated expression of neurotrophins and their receptors in the developing CNS, cell losses are sparse in the knockout mice. However, some cell loss is observed in the knockout mice. $BDNF^{-/-}$ mice display a reduction in thickness of all cortical layers (Jones *et al.*, 1994). Furthermore, their cerebellar granular layer is thinner, and the number of apoptotic cells is considerably increased (Schwartz *et al.*, 1997). The brains of $TrkB^{-/-}$ mice also display an increased number of apoptotic cells, particularly in the dentate gyrus, and also in the basal ganglia, thalamus, and cerebral cortex (Alcantara *et al.*, 1997). While the number of motor neurons is reduced in the facial nucleus and the spinal cord of $TrkB^{-/-}$ animals (Klein *et al.*, 1993), no loss of motor neurons is described in the BDNF knockout mice. Quite opposite to the e ects of neurotrophin or Trk knockout, mutation of the p75NGF receptor results in an enlarged basal forebrain due to ablation of p75NGFR-mediated apoptosis (Van der Zee *et al.*, 1996).

To summarize, there is some cell death in the CNS of neurotrophin and Trk mutants but not to an extent comparable to what is observed in the PNS. This could be explained as a consequence of a high redundancy, with CNS cells supported by more than one neurotrophic factor. In mice deficient for TrkA, there is a reduction in the number of neurons projecting from the basal forebrain to the neocortex and hippocampus (Smeyne *et al.*, 1994), but this is not observed in $NGF^{-/-}$ mice (Crowley *et al.*, 1994), indicating that other factors (NT-3) can bind to TrkA and compensate for the lack of NGF. The CNS of BDNF/NT-4 double knockouts does not display any additional cell loss compared with the single knockouts (Liu *et al.*, 1995). The TrkB/TrkC double knockouts die within the first postnatal day, and no increased apoptosis is reported in their CNS (Silos-Santiago *et al.*, 1997). However, mice homozygous for one receptor ($TrkB^{-/-}$ or $TrkC^{-/-}$) and heterozygous for the other ($TrkB^{+/-}$ or $TrkC^{+/-}$) live considerably longer and can be studied up to postnatal day 12. At postnatal days 9 and 12, these mice display increased apoptosis in the hippocampus and cerebellum (Minichiello & Klein, 1996). Thus there is a redundancy between TrkC and TrkB in the CNS, but the cells do not become dependent on TrkB/C-mediated survival until postnatal development. Another study demonstrates that thalamic neurons are dependent on target-derived support from the neocortex during part of their development. During a

limited time window in late embryonic and early postnatal development, cell death in the thalamus of TrkB$^{-/-}$ mice is increased compared with wild-type littermates. Furthermore, blocking BDNF in the neocortex of wild-type mice during this period increases thalamic cell death (Lotto *et al.*, 2001).

There are problems with interpreting data from neurotrophin or neurotrophin-receptor knockout mice. With the exception of the NT-4 and p75NGFR mutants, the single mutants display severe cell losses in the PNS, and die within a couple of weeks after birth. Thus their general viability is a ected, and in particular malnourishment might cause secondary changes in the CNS. Furthermore, other potential e ects in the CNS will not have time to develop due to the mutant's premature death. To overcome these e ects, investigators have developed new animal models, conditional knockouts, where the investigated genes are knocked out later during development, or in particular cell populations. In one study in which TrkB was knocked out in the neocortex pyramidal neurons, increased death of these cells was observed between six and ten weeks of age (Xu *et al.*, 2000). Contrary to this, a study of mice with a conditional knockout of TrkB in the CNS did not describe any increased cell death in the neocortex (Medina *et al.*, 2004). Apart from addressing the neurotrophins' role as neuronal survival factors, the conditional knockouts have expanded our knowledge of other neurotrophin functions during CNS development.

Neurotrophins affect neuronal differentiation and proliferation

As mentioned above, neurotrophins activate several signaling pathways, including pathways that govern cell di erentiation (Reichardt, 2006). In line with this, neurotrophins have been shown to act on immature cells, such as stem and precursor cells. This has been demonstrated in cell lines, in primary cultures and in vivo. Primary cultures of neural stem cells (pluripotent cells capable of asymmetrical division) from the brain or neuronal crest di erentiate in response to added neurotrophins (Sieber-Blum *et al.*, 1993). The neurotrophins can both promote and inhibit di erentiation. In the dorsal root ganglia of NT-3 knockout mice, precursor cells di erentiate prematurely into sensory neurons (Farinas *et al.*, 1996). The CNS of TrkB knockout mice displays reduced expression of cortical markers, indicating an impaired neuronal di erentiation, although this might be secondary to positional e ects due to altered migration of the newborn cortical cells (Medina *et al.*, 2004). These mice also display a reduced myelination, in line with recent evidence that neurons dictate whether they will become myelinated or not, signals that are transmitted via their Trk receptors (Chan *et al.*, 2004). BDNF is also capable of regulating neuronal expression of transmitter substances and neuropeptides, in particular neuropeptide Y (NPY) (Jones *et al.*, 1994; Nawa *et al.*, 1994).

Sometime during di erentiation, a cell will become post mitotic. Thus there is a connection between di erentiation and proliferation. Some studies have implicated the neurotrophins as regulators of proliferation. NGF and NT-3 increase proliferation of neuroblasts derived from rat embryonic dorsal root ganglia (Memberg & Hall, 1995). An opposite e ect is observed in cultured cortical precursor cells, which respond to added NT-3 by reduced proliferation and increased neuronal di erentiation (Ghosh & Greenberg, 1995). In utero electroporation in mice of TrkB and TrkC sh-RNA, or BDNF mRNA, demonstrates that BDNF and TrkB/C signaling regulates neural precursor proliferation and di erentiation during cortical development. Receptor knockdown by TrkB sh-RNA decreases, while addition of BDNF increases, proliferation and neurogenesis (Bartkowska *et al.*, 2007). However, no e ect on proliferation is described in mice with a conditional CNS knockout of TrkB (Medina *et al.*, 2004). While the approach in the first study only decreases TrkB levels, the second study results in an earlier and more complete abolition of TrkB. It is therefore possible that the TrkB knockout cells activate compensatory pathways, which could explain the di erent results.

Neurotrophins affect cell migration

An important aspect of CNS development is cell migration. This process is well integrated with cell proliferation and di erentiation. The cells of the future brain are born in proliferative zones, and then migrate to their final destinations, often di erentiating along the way. It is therefore not surprising that factors capable of regulating proliferation and di erentiation also regulate cell migration. Cultured embryonic cortical neurons have been shown to migrate along a gradient of NT-4 or BDNF, a process that is mediated via the TrkB receptor (Behar *et al.*, 1997). A role for neurotrophins in cell migration has also been demonstrated in vivo. In the neocortex, cells enter the developing cortical plate

by either radial or lateral migration. Neurons projecting out from the brain are born in the ventricular region and enter the developing cortical plate by radial migration (Rakic & Caviness, 1995). γ-Aminobutyric acid (GABA)ergic interneurons are born in the ganglionic eminence (Ang *et al.*, 2003), the early born Cajal-Retzius cells (a transient cell population involved in the regulation of radial migration) in the cortical hem (Yoshida *et al.*, 2006). These cells enter the cortical plate via lateral migration. Application of NT-4 to cortical slice cultures, or intraventricular injection in mouse embryos, greatly increases the number of Cajal-Retzius and GABAergic cells that migrate into the marginal zone of the cortical plate. BDNF has a similar e ect, but is less than tenfold as e ective in this system (Brunstrom *et al.*, 1997). However, both BDNF and NT-4 are equally potent in inducing lateral migration of green fluorescent protein (GFP)-labeled cells from E14 to E16 ganglionic eminence explants to the interstitial and marginal zone of cortical explants of the same age (Polleux *et al.*, 2002). This is demonstrated as a direct e ect, mediated by the PI3k pathway.

In transgenic mouse embryos that overexpress BDNF in neural stem and precursor cells, lateral migration is disturbed, and the normal distribution of Cajal-Retzius cells as a band close to the pial surface is replaced by clusters of Cajal-Retzius cells interspersed by GABAergic cells (Ringstedt *et al.*, 1998; Alcantara *et al.*, 2006). In addition to these direct e ects on tangential migration, radial migration and thereby cortical lamination are disturbed. This can in part be explained as a secondary e ect, since BDNF is a negative regulator of Reelin expression by Cajal-Retzius and other cortical plate cells (Ringstedt *et al.*, 1998; Alcantara *et al.*, 2006). Reelin is a substance involved in the regulation of radial migration (D'Arcangelo *et al.*, 1995; Ogawa *et al.*, 1995). In addition, BDNF is a survival factor for Cajal-Retzius cells (Ringstedt *et al.*, 1998), and a ects expression of axon guidance receptors Robo1 and Robo2, which can regulate cell migration (Alcantara *et al.*, 2006; Andrews *et al.*, 2006). The neocortex of the conditional CNS TrkB knockout mice also displays disturbed lamination. This is proposed to be due to a delay in radial migration, mainly mediated via the Ras/MAPK and PI3/Akt signaling pathways (Medina *et al.*, 2004). In sum, there is ample evidence that the TrkB ligands NT-4 and BDNF contribute to the regulation of both lateral and radial cell migration within the CNS, both directly and by a ecting the expression of other agents.

Neurotrophins affect neurite outgrowth in the CNS

Wiring of the brain is an important aspect of neural development. Axons have to be sent out to reach distant or nearby targets, dendrites have to branch into dendritic trees, and proper contact between axons and dendrites has to be made. Neurotrophins are known to a ect neurite outgrowth. An early study demonstrated that NT-3 treatment of lesioned corticospinal tracts in adult rats induced the lesioned axons to send out collateral branches (Schnell *et al.*, 1994). Neurotrophins can also induce turning of growth cones in vitro (Ming *et al.*, 1997; Song *et al.*, 1997). Naturally, this suggests that they can act as axon guidance factors in vivo. In support of this, sensory axons in mouse embryos target implanted beads coated with NGF, BDNF, or NT-3 (Tucker *et al.*, 2001). Moreover, the neurotrophins promote elongation of the sensory axons. Di erentiated e ects of neurotrophins on neurite outgrowth have been demonstrated in slice cultures of postnatal ferret visual cortex. Addition of BDNF increases dendritic length and branching in layer 4, while NT-4 has a similar e ect on neurons in layers 5 and 6 (McAllister *et al.*, 1995). Blocking BDNF function with antibodies decreases dendritic arborization in layer IV, while blockade of NT-3 function increases it. In layer VI, the e ects of blocking BDNF or NT-3 are reversed (McAllister *et al.*, 1997).

A study of BDNF$^{-/-}$ mice described altered dendritic arborization in cerebellar Purkinje cells (Schwartz *et al.*, 1997). Neocortical layers of adult (28 week) CNS TrkB knockout mice are compressed, the neurons tightly packed, and their dendrites not fully di erentiated (Medina *et al.*, 2004). The neocortices from mice with a conditional TrkB knockout in pyramidal neurons also display compressed layers. Biocytin labeling of individual pyramidal neurons reveals that TrkB-deficient cells have thinner dendrites and a reduced dendritic complexity compared with wild-type neurons (Xu *et al.*, 2000).

Neurotrophins are also involved in the establishment of thalamocortical connections. Mice deficient for p75NTR display a diminished thalamic innervation of visual cortex (McQuillen *et al.*, 2002). Interestingly, this is not due to a direct e ect on the axonal pathfinding, since the thalamic neurons that innervate visual cortex in wild-type mouse embryos do not express p75NTR . However, p75NTR is expressed by the early subplate neurons from the neocortex to

the internal capsule. These are pioneer neurons that act as a sca old for early thalamocortical neurons on their way to the neocortex (De Carlos & O'Leary, 1992). Most subplate neurons in the p75NTR-mutant mice project correctly, but their growth cones are highly reduced in complexity. Interaction between the growth cones of the two cell populations has been suggested as important for the subplate neurons' role as a sca old. Therefore, the subplate neurons' aberrant growth cones might cause the thalamic neurons to misroute on their way to visual cortex (McQuillen *et al.*, 2002).

Moreover, mice in which NT-3 is ablated in the embryonic telencephalon display reduced thalamocortical projections to the visual and retrosplenial cortex (Ma *et al.*, 2002). Thalamocortical projections arrive in the subplate as in wild-type embryos, but the axons fail to send collaterals into the cortical plate, which normally express NT-3 during this stage of development. Later during development, the thalamocortical projections retract, but this is not connected with increased death of thalamic neurons. The authors argue that NT-3 most likely exerts a short-distance tropic (axon-guiding) e ect on the thalamocortical neurons. Behavioral studies demonstrate that the mice have an impaired visual function (Ma *et al.*, 2002). NT-3 (and BDNF) also promotes axon growth in cultured thalamic explants (Hanamura *et al.*, 2004). In line with this, NT-3 expression in the embryonic neocortex is high during thalamic innervation but declines later during postnatal development. BDNF expression, on the other hand, is low in the embryonic neocortex, but increases during postnatal development. Neuronal plasticity in the adult brain is influenced by BDNF.

Neurotrophins affect synaptic plasticity

During development and adulthood, the brain has to adapt and modify itself in response to external stimuli. This is largely done by altering the number and the strength of the synaptic connections – synaptic plasticity. Synapses that are in frequent use are strengthened, while those that are not eventually disappear. Neurotrophins, in particular BDNF, are important in this activity-dependent modulation of synapses. Neuronal activity is known to induce expression of neurotrophins by the neurons (Ernfors *et al.*, 1991). In the mature brain, neurotrophin-regulated plasticity is involved in memory formation (long-term potentiation [LTP]) in the hippocampus and the neocortex (Korte *et al.*, 1995; Figurov *et al.*, 1996; Akaneya *et al.*, 1997; Jiang *et al.*, 2001) (for a review, see Lu *et al.*, 1989). An example of plasticity in the developing brain is the formation of visual cortex ocular dominance columns. This occurs during postnatal development, when axons from the visual system enter layer IV of the visual cortex. Here their terminals are separated in eye-specific ocular dominance columns. Manipulation of the TrkB input in kittens, by addition of either exogenous BDNF or NT-4, or by a TrkB antagonist, inhibits the formation of ocular dominance columns. BDNF is expressed in the visual cortex during postnatal development and adulthood, and the level of BDNF expression is proportional to the level of visual input (Castren *et al.*, 1992). Blocking one eye (monocular deprivation) reduces BDNF levels in the contralateral visual cortex (Bozzi *et al.*, 1995). Monocular deprivation during postnatal development alters the size of the ocular dominance columns in favor of the active eye, but only if it occurs during a certain time window. This is called the critical period for ocular dominance plasticity. Dark-rearing animals can delay this time window into adulthood. Overexpression of BDNF in the postnatal neocortex of transgenic mice shortens the critical period for ocular dominance plasticity and accelerates maturation of the visual cortex (Huang *et al.*, 1999). Furthermore, the BDNF-overexpressing mice do not have their plasticity window delayed by dark-rearing (Gianfranceschi *et al.*, 2003). Interestingly, this indicates that visual experience modulates visual cortex build-up by regulating BDNF expression, and that BDNF overexpression can replace the influence of visual experience in this system.

BDNF in adult life and in mental illness

Expression of BDNF and its TrkB receptor is high and widespread in the adult brain, and BDNF is involved in memory formation and other forms of plasticity. There are also indications of BDNF being a factor in mental illness, conditions that often are described as having a developmental background. The following discussion provides some highlights of the role of BDNF in adult plasticity and mental illness.

As described above, BDNF is a regulator of synaptic strength and dendritic arborization. In line with this, BDNF is present in the neuronal processes. Interestingly, visual stimulation a ects BDNF localization in the dendrites of visual cortex pyramidal cells (Tongiorgi *et al.*, 2006). Neuronal activity increases

BDNF secretion, but for a long time it was not possible to study the function of active, as opposed to constitutive, regulation (Lu, 2003). However, a recent study utilized a polymorphism in the *BDNF* gene (Egan *et al.*, 2003). Valine (val) was replaced by methionine (met) at position 66 in the pro-region of the gene. Labeled constructs of val-BDNF and met-BDNF were transfected into rodent hippocampal neurons. The val-BDNF protein was distributed in a punctuate pattern throughout the cell soma and the neuronal processes, whereas the met-BDNF protein was clustered in the perinuclear region. It was further shown that neuronal activity stimulates val-BDNF release to a much higher degree than met-BDNF release, while constitutive secretion is similar. This di erence turns out to be important for brain function, since human subjects with a met/met genotype display an impaired hippocampus-dependent episodic memory compared with subjects with the val/val genotype (Egan *et al.*, 2003; Lu, 2003). On the other hand, the met-BDNF polymorphism is associated with a less neurotic personality type (significantly lower mean neuroticism score) (Sen *et al.*, 2003). Furthermore, the val-BDNF genotype is associated with a higher risk of bipolar disorder (Neves-Pereira *et al.*, 2002; Sklar *et al.*, 2002). Another BDNF polymorphism, C270T, has been associated with schizophrenia (Szekeres *et al.*, 2003). Both increased (Takahashi *et al.*, 2000; Iritani *et al.*, 2003) and decreased (Durany *et al.*, 2001) BDNF levels in the hippocampus of patients with schizophrenia have been reported. An increased expression has also been reported in the neocortex (Takahashi *et al.*, 2000; Durany *et al.*, 2001; Iritani *et al.*, 2003). Patients with autism display increased BDNF levels in the basal forebrain (Perry *et al.*, 2001). However, it is likely that the alterations of BDNF expression associated with schizophrenia and autism are compensatory rather than causative, although BDNF hyperactivity has been proposed as a cause of autism (Tsai, 2005).

Conclusions

The neurotrophins' classic function as target-derived survival factors is well established in the PNS. It is also clear that they can act locally to promote survival and also regulate cell di erentiation, maturation, and even apoptosis. It has been harder to demonstrate the neurotrophins' role as target-derived survival factors in the CNS. This has been explained as due to a redundancy among neurotrophic factors in the CNS, and to the fact that neurotrophin knockout mice die during early postnatal development, before the CNS neurons become dependent on neurotrophic support. Studies in mice mutant for more than one neurotrophin or neurotrophin receptor, and in conditional knockouts where inactivation is constrained temporally or to certain cell populations, have overcome these limitations. It is now clear that neurotrophins act as target-derived survival factors in the CNS. However, this is far from their only, and perhaps not even their predominant, function. There are studies demonstrating that neurotrophins regulate cell proliferation, di erentiation, and migration in the CNS. They regulate neurite extension and can act as axon-guidance factors at short range, and are thus implicated in wiring the brain. At a more local level they contribute to the emergence of neuronal networks by regulating dendritic arborizations and synaptic plasticity. In sum, neurotrophins are involved in the whole range of processes that are necessary to build a brain, from regulation of individual cells to maturation of neuronal networks.

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Historic box 3 The discovery of programmed neuronal death and the nerve growth factor
Hugo Lagercrantz

It was well known at the beginning of the twentieth century that, by removing the target organ, some neurons innervating the organ disappear. Viktor Hamburger, who started as a student working with Hans Speman (see Historic Box 1) showed that, by removing the wing of the chick embryo, the number of neurons disappeared. Hamburger gave his paper to an Italian neuroanatomist, Guiseppi Levi, who together with a student, Rita Levi-Montalcini, challenged this finding and showed that the nerves first grow normally and then degenerate when the wing is removed. Thus the target organ seemed to keep the neurons alive.

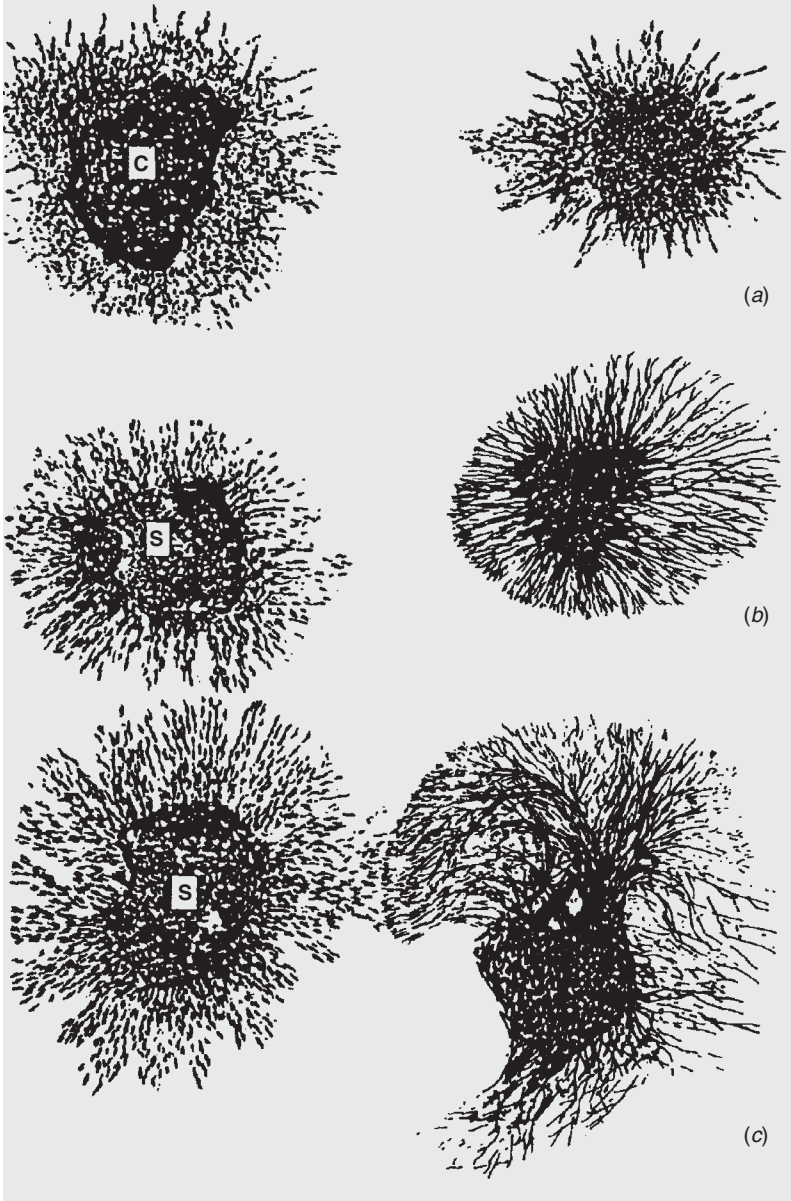

Fig. HB3 The discovery of programmed neuronal death and nerve growth (Nobel Foundation)

These studies by Levi and Levi-Montalcini were performed under remarkable circumstances. These researchers were barred from working in universities in Mussolini's Italy, as they were Jewish. Therefore, Rita Levi-Montalcini set up a laboratory in her bedroom "with a few indispensable pieces of equipment, such as an incubator, a light, a stereomicroscope and a microtome. The object of choice was the chick embryo, and the instruments consisted of sewing needles transformed with the help of a sharpening stone into microinstruments. My Bible and inspiration was an article by Viktor Hamburger … ." In July 1942, the heavy bombing of Turin forced Rita Levi-Montalcini to move to a small country house, where she continued her experiments. Due to food shortages, the eggs were scrambled for food after the experiments.

After the war Rita Levi-Montalcini was invited by Viktor Hamburger to St Louis in the United States to continue her research, where she stayed for several decades. A series of experiments was conducted, first with Hamburger and then with the biochemist Stanley Cohen, which led to the discovery of the nerve growth factor (NGF). A crude extract was first isolated from a mouse tumor and shown to produce the famous halo e ect when incubating a naked ganglion cell. When trying to further purify NGF, a snake venom was used, but instead of degrading NGF it promoted the halo e ect. In this way, the salivary glands were discovered to be a rich source of NGF. Rita Levi-Montalcini shared the Nobel Prize in physiology or medicine with Stanley Cohen in 1986.

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Chapter 7

Neurotransmitters and neuromodulators

Eric Herlenius and Hugo Lagercrantz

Introduction

Neuronal communication is mainly mediated by myriads of synapses via neurotransmitters, although there are also electrical synapses. Neurotransmitters can be defined as chemicals released from neurons that act on specific receptors. However, neuromodulators released from other cells, such as adenosine triphosphate (ATP), adenosine, and prostaglandin, can also affect neuronal signaling. Neurotransmitters and their receptor subtypes are expressed in high amounts during certain stages of development, but then persist in only a few synapses (Parnavelas & Cavanagh, 1988).

The transient increase in expression of a transmitter and receptor subtype in the central nervous system (CNS) may occur during a susceptible developmental time window. (A transient increase occurs, but it may not always occur during a critical window.) Development is characterized by timing and spacing or critical periods when some kind of stimulation is essential for correct development. The transmitters and modulators affect formation of synaptic contacts, maturation of synapses, and structural refinement of connectivity by regulating electrical activity, excitability, and release of neurotrophins (Zhang & Poo, 2001). In particular, at birth a cascade of neurotransmitters and transcriptional factors is activated. For example, the norepinephrine surge at birth may be important for initiating the bonding of the infant to the mother by increasing the ability to sense odors (Sullivan *et al.*, 1994). Imprinting at birth and visual input to form the ocular dominance columns also occur during critical periods, and are probably dependent on the switching on and off of neurotransmitters. However, no critical period ends suddenly; rather it tapers off gradually. Expression of transmitters and receptor subtypes is critical for the neuronal differentiation, development of synapses, and formation of neuronal networks underlying behavior in the fetus as well as in the growing child and adult human.

The general role of neurotransmitters in the making of the brain is far from clear. Several studies support the importance of and an early role for neurotransmitter signaling before synaptogenesis. Although it can be postulated that neurotransmitters are involved in the detailed wiring of the neuronal circuits, it has been demonstrated that knocking out of all synaptic tra cking in the mouse does not prevent normal brain assembly, including formation of layered structures, fiber pathways, and morphologically defined synapses (Verhage *et al.*, 2000). However, transmitters and synaptic activity are necessary for survival of synaptic contacts, as without vesicle release of transmitters neurons undergo apoptosis after formation of synapses. This is because their maintenance depends on neurotransmitter secretion. In addition, it was recently shown that early release of transmitter is unconventional in not requiring action potentials, Ca^{2+} entry, or vesicle fusion, which may explain how early synapses and fiber tracts are formed (Demarque *et al.*, 2002; Owens & Kriegstein, 2002a). Intercellular connections, via gap junctions, and release of neuromodulators such as ATP through unopposed gap junctional channels, "connexins," have emerged as central components in intercellular signaling networks vital for the embryo and the developing CNS. This chapter focuses on the chemical transmitters and modulators with membrane receptors. For reviews of the connexins see, for example, Trosko (2007), Andang and Lendahl (2008), and Elias and Kriegstein (2008).

Markers for neurotransmitters and neuromodulators during CNS development generally appear first in

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the caudal and phylogenetically older part of the brain, probably due to earlier neurogenesis (see Semba, 2004). The neurotransmitters or modulators can act on either metabotropic or ionotropic receptors (see, e.g., Cooper *et al.*, 2003). The action of the metabotropic receptors is based on their effects on G- or N-proteins and their associated enzymes and channels in the lipid bilayer of the membrane. These effects are slower (tens of milliseconds) than for the ionotropic receptors. Epinephrinergic, muscarinic, and peptidergic receptors are often metabotropic and have a more modulatory role in the mature CNS. The ionotropic receptors respond rapidly and are also termed class I receptors. They act on ion gates, which they can open or close in less than a millisecond. The ion channels consist of transmembrane proteins, which can be selective for cations (activatory receptors) or anions (inhibitory). The nicotinic acetylcholine ligand-gated ion channel is a prototype (Changeux & Edelstein, 2005). The binding of a ligand cause an allosteric change in the ion channel pore. The ionotropic nicotinic acetylcholine receptor (nAcR), the -aminobutyric acid $(GABA)_AR$, the glycine receptor (GlyR), and the 5-hydroxytryptamine $(5\text{-}HT)_3$ receptor are members of the same evolutionary superfamily and have a similar structure.

The 7-transmembrane receptors (7-TMRs) are the most important metabotropic receptors. They are also allosteric and G-protein-coupled (GPCR) and represent by far the largest family of receptors. These receptors account for more than 1% of the human genome and almost a third of all drugs on the market act via these receptors. The β_2-adrenoreceptor is a kind of prototype. It has been cloned and recently also crystallized (Rasmussen *et al.*, 2007).

Small lipophilic or gaseous molecules that penetrate the cell membrane have during the past decade proved to be important neuroactive agents. These unconventional transmitters are not stored in synaptic vesicles and do not act at conventional receptors on the surface of adjacent neurons. Rather they may interact with nuclear receptors, e.g., retinoids, vitamin D (Mangelsdorf *et al.*, 1995), or enzymes in the cytosol (guanylyl cyclase), e.g., nitric oxide (NO) and carbon monoxide (CO). However, their roles during development of the CNS are still under investigation and they are not discussed here. Properties of these "atypical" neural modulators have recently been reviewed by Boehning and Snyder (2003) and Li and Moore (2007).

Ontogeny of neurotransmitter systems

The choice of neurotransmitter of a precursor neuron depends on the environment. In a series of remarkable experiments Nicole Le Douarin demonstrated that when the crest of the sympathetic trunk from a quail was transplanted into the vagal region of a chick host, the nerves became cholinergic (Le Douarin, 1981). Conversely, when vagal neurons were transplanted into the sympathetic trunk, the nerves became epinephrinergic. The type of neurotransmitter expressed seemed to be dependent on a tissue factor. When cells from the sympathetic ganglia were cultivated in a medium from a heart cell culture, the epinephrinergic neurons became cholinergic (Patterson & Chun, 1977). The choice of transmitter could also be affected by the presence of corticosteroids in the medium. Thus, environmental factors are important for differentiation and may have an inductive role during critical stages of development.

The catecholamines

The catecholamine-synthesizing enzyme tyrosine hydroxylase has been detected on the first day of/after incubation of the chicken, dopamine has been detected on the second day and norepinephrine and epinephrine on the third day. High concentrations of catecholamines have been detected in Hensen's node, corresponding with the notochord of the mammalian embryo (see Pendleton *et al.*, 1998). Catecholaminergic neurons are generated at the time of telencephalic vesicle formation in rodents as well as in primates. The monoaminergic neurons reach the cerebral wall as cortical neurogenesis begins. Catecholamines have a crucial role in early development, which has been demonstrated by deleting the genes encoding for tyrosine hydroxylase (Zhou *et al.*, 1995) and dopamine β-hydroxylase (DBH) (Thomas *et al.*, 1995).

Norepinephrine

Norepinephrine is assumed to be involved in arousal and attention, fear and anxiety, and learning and memory. The cell bodies of the norepinephrinergic neurons are concentrated in the brainstem, particularly in the locus ceruleus (A6) within the caudal pons (Fig. 7.1). From this structure five major norepinephrinergic tracts originate which project to virtually all regions of the brain (Goridis & Rohrer, 2002). There

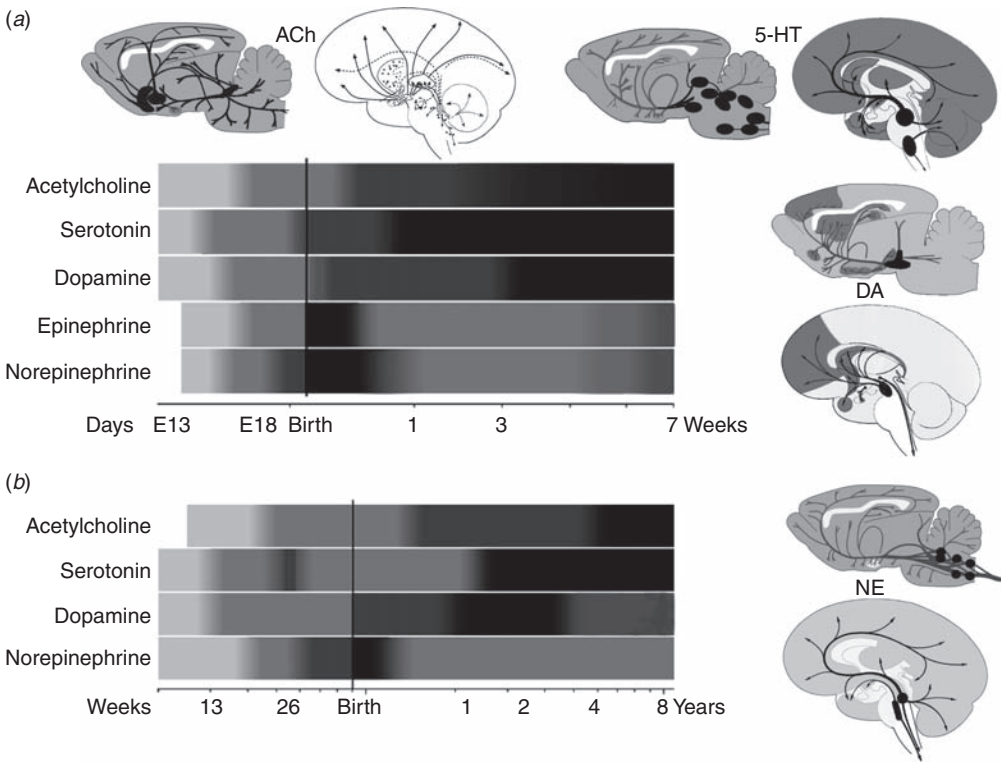

Fig. 7.1 Arbitrary levels of monoamines and acetylcholine in (*a*) rat and (*b*) humans versus age (10-logarithmic scale). Sagittal illustrations of cell bodies and projections of monoamine neurotransmitter systems: acetylcholinergic (ACh), dopaminergic (DA), serotonergic (5-HT), and norepinephrinergic (NE) pathways in the rat and human brain, and epinephrinergic pathways in the rat. Embryonic age (E) expressed in days for rats and weeks for humans, postnatal age in weeks (rats) and years (humans). (Data from Olson & Seiger, 1972; Herregodts *et al*., 1990; Naeff *et al*., 1992; Sundstrom *et al*., 1993; Almqvist *et al*., 1996). (Brain maps modified with permission from Heimer, L. (1988). *The Human Brain and Spinal Cord*. New York: Springer Verlag; and Zigmond, M. J., Bloom, F. E., Landis, S. C., Roberts, J. L., & Squire, L. R., eds (1999). *Fundamental Neuroscience*. San Diego: Academic Press.)

are also clusters of norepinephrinergic cell bodies in, for example, the nucleus tractus solitarius (A2), and in the lateral ventral tegmental field (A5). Fibers from these nuclei intermingle with those from the locus ceruleus. Norepinephrinergic neurons appear at an early stage in the CNS, at 12–14 days in the rat (Olson & Seiger, 1972) (total gestational age 21 days), and 5–6 weeks in the human (Sundstrom *et al*., 1993) (see Fig. 7.1).

Norepinephrine is essential for normal brain development. The norepinephrinergic system regulates the development of the Cajal-Retzius cells, which are the first neurons to be generated in the cortex and are proposed to be instrumental in neuronal migration and laminar formation (Naqui *et al*., 1999). Furthermore, α_{2A} receptors are expressed by migrating neurons in the intermediate zone, characterized by a spindlelike shape, radial alignment, and close association with radial glia (Wang & Lidow, 1997). The radial glia participate in key steps of brain development, cortical neurogenesis, and migration (Noctor *et al*., 2001, 2004). Thus, epinephrinergic transmission may be involved in regulating the generation, migration, and maturation of cerebral cortical cells.

Administration of 6-hydroxyl-dopamine prevents the natural programmed cell death of the newborn neurons and delays the formation of cortical layers. Lesioning the norepinephrinergic projections or blocking neurotransmission with receptor antagonists prevents astrogliosis and glial cell proliferation. Depletion of norepinephrine during the perinatal period results in subtle dendritic changes and possibly also alterations in cortical differentiation (see Berger-Sweeney Hohmann, 1997).

The role of norepinephrine has been investigated by targeted disruption of the *DBH* gene (Thomas *et al*., 1995). This resulted in fetal death, probably due to cardiovascular failure. Only about 5% of the homozygotic

mice survived until adulthood, presumably due to some placental transfer of norepinephrine. Most of the mice could be rescued to birth by providing them with dihydroxyphenylserine (DDPS), a precursor that can be converted to norepinephrine in the absence of DBH. These mice showed reduced ability for acquisition and retention for some tasks. Interestingly, female mice seemed to be deficient in their ability to take care of their offspring. Thus, there seems to be a critical window during early development when norepinephrine is involved in forming the pathways responsible for maternal behavior (Thomas & Palmiter, 1997). Norepinephrine is probably also involved in the olfactory learning of the newborn, which is of importance for maternal recognition (Insel & Young, 2001). The epinephrinergic receptors are metabotropic and are subdivided into three families, α_1, α_2, and β. α_2 and β_1 dominate in the brain. There is a transient overexpression of α_2 receptors in the white matter and many brainstem nuclei during the perinatal period, suggesting a developmental role (Happe *et al.*, 2004).

Dopamine

Dopamine has a very important role in motor and cognitive programs. The cell bodies of the dopaminergic neurons are concentrated in the substantia nigra, the ventral tegmental area, and the retrorubral field, and they project to the basal ganglia, the olfactory bulbs, the limbic regions, and the hippocampus and cortex (Fig. 7.1). The prefrontal cortex in particular is rich in dopamine content, which is interesting with regard to its important role in reasoning, planning, problem solving, and coordinating performance in humans (Diamond *et al.*, 2004).

Dopaminergic neurons appear early during development, at a gestational age of 10–15 days in the rat (Olson & Seiger, 1972) and 6–8 weeks in the human (Sundstrom *et al.*, 1993) (Fig. 7.1), earlier in females than in males. The dopamine turnover is relatively high during the perinatal period when compared with that in adulthood.

There are two main types of dopamine receptors: D_1 and D_2, but there are also D_3, D_4, and D_5 receptors. Stimulation of the D_1 receptors results in an increase in cAMP formation and phosphorylation of DARPP-32, whereas D_2 receptors mediate a decrease in cAMP formation. Extremely high levels of D_1 receptors have been reported in the pallidum during the perinatal period (Boyson & Adams, 1997). D_1 receptor stimulation regulates transcription of other genes, and it is possible that abnormal perinatal stimulation can have long-term consequences (see below).

Disturbances of the development of the dopaminergic system may lead to dyskinesia, dystonia, tics, obsessive-compulsive disorders, and abnormal eye movements. This has been observed in dopamine-depleted rats after 6-hydroxyl-dopamine treatment but with preserved norepinephrine effect. Mice with deletion of the tyrosine hydroxylase gene have been shown to be hypoactive and develop adipsia and aphagia, which can be treated with L-dopa (Zhou & Palmiter, 1995).

D_1 receptors are involved in working memory performance (Williams & Goldman-Rakic, 1995). A disturbance of the development of the dopaminergic system has been postulated to contribute to the development of attention deficit hyperactivity disorder (ADHD), in which a deficient working memory is an important component of the syndrome. Perinatal exposure to a very low dose of methyl mercury (MeHg) results in neurotoxic effects, e.g., on memory retention, and such alterations of brain function persist into adult life (Dare *et al.*, 2003).

Infants with phenylketonuria and probably deficient dopaminergic innervation of the prefrontal cortex have been found to have an impaired working memory (Diamond, 1996). The catechol-*O*-methyltransferase (*COMT*) gene affects how long dopamine acts in the prefrontal cortex. It was recently shown that genotypic differences in *COMT*, inducing differences in breakdown of prefrontal dopamine, are related to differences in specific cognitive performance in normal-developing children (Diamond *et al.*, 2004; Diamond, 2007).

Epinephrine

The existence of epinephrine in the brain was not accepted until the epinephrine-synthesizing enzyme phenylethanolamine-*N*-methyl transferase (*PNMT*) was detected by immunohistochemical methods. This enzyme was localized in the lower brainstem (Fig. 7.1) intermingled with norepinephrinergic neurons. Epinephrine in the brain is probably involved in neuroendocrine and blood pressure control. During early development epinephrine contributes to enhance the fetal respiratory rhythm in rat medulla while during fetal maturation it acquires inhibitory actions on locus ceruleus and brainstem respiratory rhythm (Fujii *et al.*, 2006). *PNMT* occurs predominantly before birth in the rat CNS; after birth, there is a decline in *PNMT*-containing structures (see Foster, 1992).

Serotonin

Serotonin (5-HT) and serotonergic neurons are localized in the midbrain, the pineal gland, the substantia nigra, the hypothalamus, and the raphe nuclei of the brainstem (Fig. 7.1). The 5-HT neurons have widespread projections making it possible to coordinate complex sensory and motor patterns during various behavioral states.

There exist a multitude of heterogeneous 5-HT receptors, classified into seven main receptor subtypes, 5-HT1–7, with more than 15 molecularly identified 5-HT receptor subtypes so far. The majority of the 5-HT receptors belong to the G-protein receptor family, except for 5-HT_3 receptors, which are ligand-gated ion channel receptors (for a review see, Hoyer *et al.*, 2002). 5-HT enhances motor neuron excitability. Serotonergic tonic activity is highest during waking and arousal and absent during active or rapid eye movement sleep. If the gene encoding for 5-HT_{1B} receptors is knocked out the proportion of active sleep is increased (Boutrel *et al.*, 1999).

Serotonin can already be detected in the fertilized egg and is involved in early morphogenesis of the heart, the craniofacial epithelia, and other structures. If embryos are cultured in the presence of serotonin uptake inhibitors or receptor ligands, specific craniofacial malformations occur. Serotonergic cells in the raphe are among the earliest to be generated in the brain (about embryonal day [E]10–E12 in the mouse). After their generation in the raphe, they start to project diffusely into the spinal cord and the cortex. Serotonergic cells appear in the fifth to twelfth gestational week in the human (Fig. 7.1). These cells send axons to the forebrain and may be of importance in the differentiation of neuronal progenitors (Gaspar *et al.*, 2003). Moreover, serotonin has been reported to affect neuronal proliferation, differentiation, migration, and synaptogenesis, although knocking out serotonin receptors or genes involved in its metabolism did not seem to cause marked alterations in brain histology (Gaspar *et al.*, 2003). Excess serotonin prevents the normal development of the somatosensory cortex, which has been demonstrated in monoamine oxidase knockout mice (Cases *et al.*, 1996). At birth, serotonergic-containing axons penetrate all cortical layers, but then decline markedly after about three weeks. Depletion of serotonin after birth seems to have little effect on cortical development.

Maternal serotonin may be critically involved in fetal morphogenesis (Cote *et al.*, 2007). This was demonstrated in a mouse line deficient in peripheral serotonin biosynthesis by disruption of the tryptophan hydroxylase gene (*tph1*). The *tph1*-null females were bred with *tph* wild-type males. The offsprings displayed marked brain abnormalities. The phenotype of the mothers and not of the embryo seemed to be most important. These findings may have some implications with regard to autism, which may be related to hyposerotonism during fetal life (see below).

Disturbed development of the cerebral serotonin system has also been observed in victims of sudden infant death syndrome (SIDS). Paterson *et al.* (2006) found fewer 5-HT receptor-binding sites in regions of the medulla involved in homeostatic functions in SIDS victims than in controls. On the other hand, there were a higher number of serotonin neurons in the former group, possibly as a compensatory mechanism. A recent mouse model might help explain how altered serotonin homeostasis can be related to sudden unexpected death in infants. Using mice with reversible overexpression of 5-HT_{1A} receptors and thus excessive serotonin autoinhibition, it was shown that altered serotonin homeostasis is associated with failure to respond to environmental changes, catastrophic autonomic dysregulation, and sudden death (Audero *et al.*, 2008).

A transient uptake and storage of serotonin in developing thalamic neurons occurs during formation of somatosensory cortex in mouse because of the temporary expression of the high-a nity serotonin transporter (SERT) (Lebrand *et al.*, 1996). This 5-HT uptake and possibly the use of 5-HT as a "borrowed transmitter" seem necessary for the normal development and the fine tuning of cortical sensory maps during their critical period of development in rodents (Gaspar *et al.*, 2003). Human fetuses have a similar restricted time period of SERT expression (gestational weeks 12–14) when thalamocortical fiber tracts develop and fine tuning of cortical sensory maps occurs (Verney *et al.*, 2002). The fetal human brain, especially cortex and hippocampus, exhibits a prenatal peak (weeks 16–22) in the density of serotonin 5-HT_{1A} receptors (Bar-Peled *et al.*, 1991). Activation of the 5-HT_{1A} receptor is associated with increased neurogenesis, neural differentiation, and dendritic maturation in the hippocampus. Whether 5-HT has a direct effect on neural progenitors or an indirect effect via the glia, which express 5-HT_{1A} receptors and release S-100B (an astroglial-derived growth factor) when 5-HT_{1A} receptors are activated, is currently not known (Gaspar *et al.*, 2003).

The serotonin concentration must be neither too high nor too low during the critical period of synaptogenesis and formation of brain connections. Miswiring problems due to excess or inadequate activation of specific 5-HT receptors during development may be involved in the genesis of psychiatric disorders such as anxiety disorders, drug addiction, and autism (for a review, see Gaspar *et al*., 2003).

Autism has been suggested to be related not only to hyposerotonism during fetal life but also to hyperserotonism postnatally (Chugani, 2002). Serotonin is transiently synthesized in high levels in young children. This overactivity declines in normal but not in autistic children. Patients with a point mutation of the gene encoding for monoamine oxidase has been found to be related to antisocial behavior (Gaspar *et al*., 2003). Selective serotonin reuptake inhibitors (SSRIs) are used by 2%–4% of pregnant women, but animal and human studies are inconclusive regarding their eventual adverse effects on CNS development at therapeutic doses, although high doses may cause anatomical and behavioral changes (Simons *et al*., 2002).

Fluoxetine, the prototype SSRI, crosses the placenta and enters the fetal brain. The limbic system in particular may be affected in utero. There may be untoward subtle effects on the fetus and the newborn. Lower Apgar score, withdrawal symptoms, and lower Bayley psychomotor index scores have been reported (Lattimore *et al*. 2005).

Acetylcholine

Acetylcholine (ACh) is one of the major neurotransmitters in the brain of importance for cortical activation, attention, memory and learning, reward, and pain. It has a major role in the control of motor tone and movement and probably counterbalances the effect of dopamine (see Johnston & Silverstein, 1998; Cooper *et al*., 2003). It is of major importance for the development and control of autonomic functions. "If a single neurotransmitter is critical for consciousness, then it must be acetylcholine" according to Koch (2004) (see Chapter 23).

The cholinergic neurons in the brain are organized in local circuit cells, for example in the caudate-putamen nucleus, and in longer projection neurons to the cortex, the basal forebrain and the mesopontine tegmentum (see Semba, 1992, 2004) and Fig. 7.1. The development of cholinergic systems has been studied by analyzing markers such as ACh, the synthesizing enzyme choline acetyltransferase (ChAT) and acetylcholinesterase. The cholinergic innervation of the cortex occurs later than the monoaminergic, about E19 in the mouse and the rat and around week 20 in the human fetus. Mature levels in rodents are not reached until after eight weeks postnatally (see Berger-Sweeney & Hohmann, 1997). The concentrations of ACh reach about 20% of the adult levels at E15 in the whole brain of the rat and about 40% at day P7 (Fig. 7.2). The levels of ChAT are much lower (1% and 8%) at the corresponding ages, indicating low firing rates of the cholinergic neurons. Conversely, the receptors reach adult levels earlier. The cholinergic markers appear sooner in the pons–medulla, probably due to earlier neurogenesis in the caudal and phylogenetically older part of the brain (see Semba, 2004).

The classic cholinergic receptors – muscarinic and nicotinic receptors – undergo important changes during development (see Dwyer *et al*., 2008). A fetal subunit of the muscarinic receptor (-AChR) is replaced by an adult type (-AChR) in the muscle endplate to increase conductance (Herlitze *et al*., 1996). The nicotinic acetylcholine receptors (nACRs) consist of five subunits centered around a central pore. The $\alpha_4\beta_2$nAChR and α_7nAChRs predominate in the brain. These receptors have been detected at E12–13 in the rat brain. Increasing levels of mRNA encoding for the subunits have also been identified during the first trimester of human fetuses (Hellstrom-Lindahl *et al*., 1998). The nicotine receptors have been shown to be important for the proliferation and/or survival of neuroblasts. Excessive stimulation of nicotinic receptors seems to enhance neuronal cell death (see Changeux & Edelstein, 2005).

Nicotinic acetylcholine receptors (nAChRs) may play important roles during development and plasticity. Activation of nicotinic acetylcholine receptors promotes synaptic contacts and the wiring during a critical period of postnatal development (Maggi *et al*., 2003). This has been demonstrated in the hippocampus but may also apply for other parts of the brain. The arousal response is lower in mice lacking the β_2-subunit of the nAChRs (Cohen *et al*., 2002).

Nicotinic exposure during fetal life seems to affect β_2-containing nAChRs, and explains some of the adverse effects of maternal smoking on the offspring (Weitzman *et al*., 1992). Newborn mice exposed to nicotine in doses corresponding to the levels human fetuses sustain during moderate maternal smoking were found to breathe irregularly and had impaired arousal

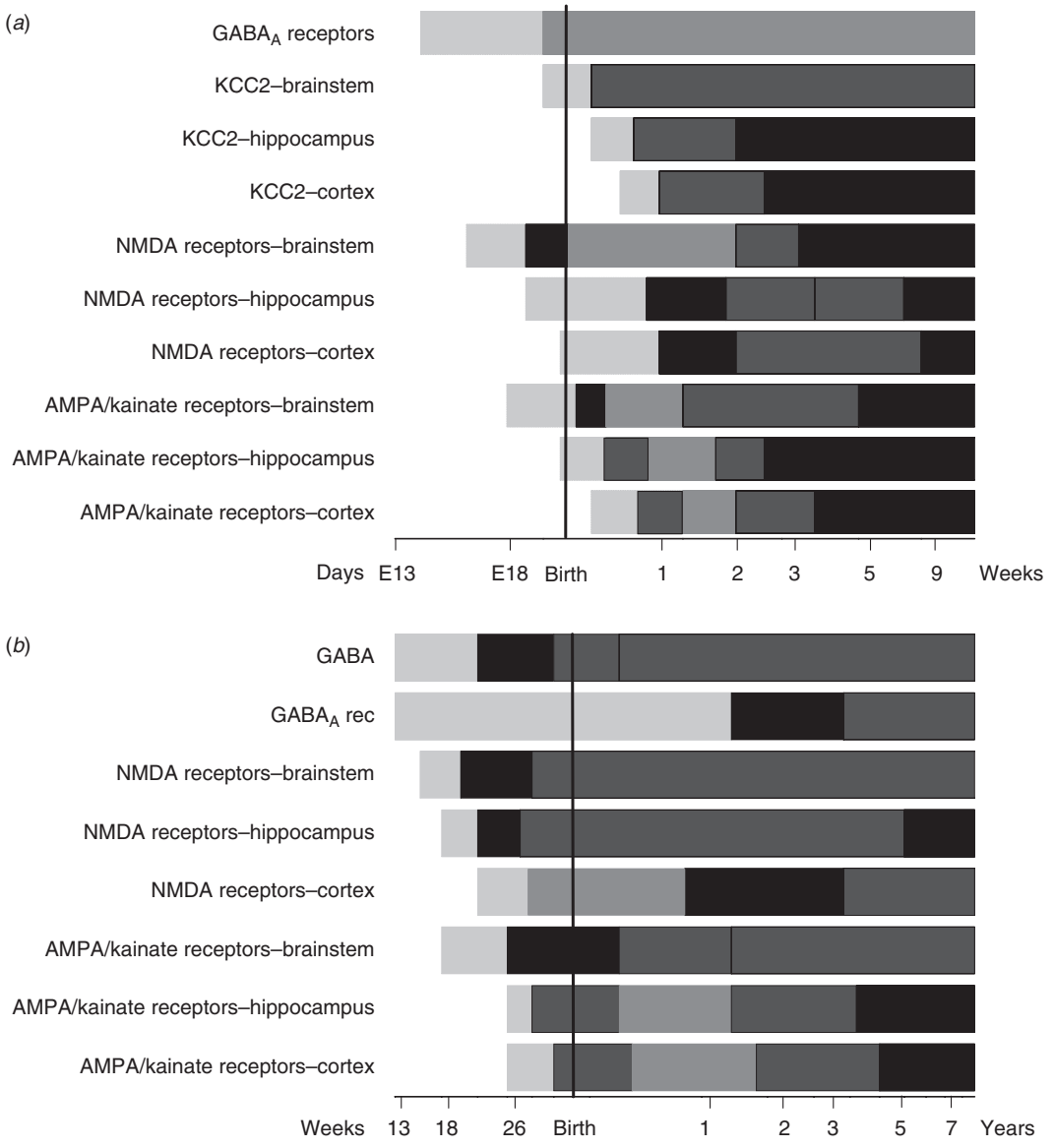

Fig. 7.2 Expression and arbitrary levels of receptors of amino acid transmitter versus age in (*a*) rat and (*b*) humans (10-logarithmic scale). The degree of shading reflects relative concentration. KCC2 is a neuron-specific cotransporter of Cl^- ions and is responsible for the switch of γ-aminobutyric acid (GABA) as an excitatory to an inhibitory neurotransmitter (see Fig. 7.1), *N*-methyl-D-aspartate (NMDA) receptors are expressed relatively earlier than the kainate and α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) receptors. It is assumed that the NMDA receptors are more involved in the wiring of the brain while the kainate and AMPA receptors are responsible for the fast traffic in the more mature brain. Embryonic age (E) expressed in days for rats and weeks for humans, postnatal age in weeks (rats) and years (humans). (Data from Hagberg *et al.*, 1997; Herschkowitz *et al.*, 1997; Johnston & Silverstein, 1998; Rivera *et al.*, 1999).

responses and catecholamine biosynthesis (Cohen *et al.*, 2002). Remarkably similar effects were seen in transgenic pups lacking the β_2-subunit (Cohen *et al.*, 2005). Thus, autonomic and catecholamine biosynthesis disturbances may contribute to the many-fold increase in risk of SIDS with maternal smoking. The development of the dopaminergic system and increase in the risk of attention deficit disorders in the offspring may also be affected (Pauly & Slotkin, 2008).

Amino acid transmitters

Amino acid transmitters are the most abundant transmitters in the CNS. However, they were recognized as

neurotransmitters in the mammalian brain much later than the monoamines and acetylcholine. This was probably due to the fact that they are involved in intermediate metabolism and constitute the building blocks of the proteins.

The amino acids are involved in the main nervous processes in the brain such as sensory input, encoding of memories, and mediating movements. In the developing brain they seem to play an important role in the wiring of neuronal networks and building of the CNS cytoarchitecture (Ben-Ari *et al.*, 1997; Wang & Kriegstein, 2008) (Fig. 7.2).

Glutamate and aspartate

Glutamate and aspartate are the dominating excitatory amino acids (EAA) and the primary neurotransmitter in about half of all the synapses in the mammalian forebrain. They constitute the major transmitters of the pyramidal cells, the dominating neurons in the cortex. This has been demonstrated by injection of radioactive labeled D-[^{3}H]glutamate into the appropriate projection areas (see Cavanagh & Parnavelas, 1988). EAA pathways undergo striking developmental changes, involving transient overshoots, especially during critical periods as evidenced in the visual cortex and hippocampus. EAA terminals are overproduced during the early postnatal period, for example after 7–14 days in the rat cortex and after 1–2 years in the human cortex, which may be related to the high generation of synapses during those periods (Bourgeois, 2002; Benitez-Diaz *et al.*, 2003) (see Fig. 7.2).

Glutamate acts on at least five types of receptor. The slower-acting metabotropic receptors, of which eight subclasses are hitherto known, are expressed at a relatively early stage. Of the ionotropic receptors, the *N*-methyl-D-aspartate (NMDA) receptors dominate in the immature brain when synaptic transmission is weak and extremely plastic (Fig. 7.2). The NMDA receptors permit entry of Na^+ and Ca^{2+} when opened. NMDA channels seem to be crucially involved in the appearance of long-term potentiation (LTP) and synaptic plasticity underlying learning and memory storage throughout life. During critical periods of development and synaptogenesis NMDA receptors play an essential role in activity-dependent plasticity and synaptic refinement (for reviews see McDonald & Johnston, 1990; Qu *et al.*, 2003; Wang & Kriegstein, 2008). Dark rearing or blocking the activity with tetrodotoxin results in preservation of the NMDA receptors in the visual cortex. Dark rearing also preserves the immature form of the NMDA receptors containing the NR2B subunit, and the expression of NR2A is delayed. This subunit switch is essential for development of rapid synaptic transmission (Fox *et al.*, 1999). During maturation, the AMPA and kainate ionotropic receptors predominate and carry most of the fast neuronal tra c in the brain. NMDA receptors are more active during early life, due to the expression of different receptor subunits, e.g., the more immature NR2B receptor subunit, allowing enhanced activation of the channel, increasing its capability to strengthen synapses and to learn (Tang *et al.*, 1999). Alas, this dominant role of NMDA and increased Ca^{2+} in ux/activation also causes the brain to be more sensitive to excitoxicity (excessive release of glutamate) caused by pre- and perinatal asphyxia (see Chapter 17). NMDA receptor stimulation by excessive glutamate release leads to Ca^{2+} in ux, which may induce subsequent neuronal apoptosis. Excess activation of NMDA and non-NMDA receptors is implicated in the pathophysiology of brain injury in several clinical disorders to which the developing brain is susceptible, including hypoxia–ischemia and seizures (McDonald & Johnston, 1990; Qu *et al.*, 2003). Fetal rats exposed to NMDA antagonists have been found to have excessive apoptosis in the same way as the asphyxiated perinatal brain. Ethanol is an NMDA antagonist and excessive inhibition of NMDA receptors causes apoptosis that may play an important role in fetal alcohol syndrome – this is also discussed below (Olney *et al.*, 2002). Thus, either too much or too little NMDA receptor activity can be life-threatening to developing neurons (Lipton & Nakanishi, 1999).

γ-Aminobutyric acid

GABA is the dominating neurotransmitter in the nonpyramidal cells, as demonstrated by uptake of [^{3}H]-GABA and immunochemical labeling of the GABA-synthesizing enzyme glutamic acid (GAD). Perhaps 25%–40% of all nerve terminals contain GABA. In lower mammals, the vast majority of the GABAergic interneurons arise in the ganglionic eminence, a subcortical area, and then migrate tangentially to their target areas in neocortex (Marin & Rubenstein, 2001). In humans, the majority of neocortical GABAergic neurons arise locally in the ventricular and subventricular zone, while proportionally fewer GABAergic neurons originate from the ganglionic eminence of the ventral forebrain (Letinic *et al.*, 2002). GABA is regarded as the main inhibitory transmitter in the

mature animal, but has a different and critical role during early development.

Before synapse formation the action of GABA is mediated through paracrine/autocrine signaling and then through classic synaptic signaling. GABA receptor function regulates embryonic and neural stem cell proliferation and differentiation (Andang *et al.*, 2008; Andang & Lendahl, 2008). During early brain development it acts as a trophic factor to in uence events such as proliferation, migration, differentiation, synapse maturation, and cell death (Owens & Kriegstein, 2002b). Moreover, GABA-induced depolarization is necessary for proper excitatory synapse formation and dendritic development of newborn cortical neurons and provides an activity-dependent mechanism for achieving the balance between excitation and inhibition in the developing cortex (Wang & Kriegstein, 2008).

GABA is also a crucial transmitter in the human infant. When vitamin B_6 was excluded from infant formula by mistake, it resulted in a disastrous series of deaths mainly due to GABA deficiency, which resulted in fatal seizures (Frimpter *et al.*, 1969). There are two types of GABA receptor: $GABA_A$ and $GABA_B$. The $GABA_A$ receptor ($GABA_A$-R) is an ionotropic receptor that gates a chloride channel. It is a transmembrane protein built of several subunits where, for example, benzodiazepines, barbiturates, and ethanol can bind to specific sites and modulate the opening properties of the chloride channel. Depending on their subunit composition, these receptors exhibit distinct pharmacological and electrophysiological properties (Sieghart & Sperk, 2002) The $GABA_B$-R is coupled to a G-protein, is present in lower levels in the CNS than the $GABA_A$ receptor, and starts to function late in CNS development.

During early development the Cl^- concentration is high in the nerve cells. When GABA opens the Cl^- channels, a depolarization (i.e., excitation) occurs. During maturation the Cl^- concentration decreases which results in an opposite effect of GABA, i.e., Cl^- ions are pumped out and the cell becomes hyperpolarized (Fig. 7.2). In this way GABA switches from an excitatory to an inhibitory neurotransmitter (Miles, 1999). This switch is due to the expression of the K^+/Cl^- cotransporter (KCC2) – reported to be expressed around birth in the brainstem, one week after birth in the hippocampus and between one and two weeks in the cortex of the rat (Miles, 1999; Rivera *et al.*, 1999; Blaesse *et al.*, 2009) (Fig. 7.2).

Thus, GABA operates mainly as an excitatory transmitter on immature neurons. As described above, glutaminergic synapses initially lack functional α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) receptors and the NMDA channels are blocked by Mg^{2+} at resting membrane potentials. GABA depolarizes immature neurons, which may result in Ca^{2+} in ux by removing the Mg^{2+} blockage of NMDA channels. Thus, $GABA_A$ receptors play the role conferred to AMPA receptors in the more mature CNS (Ben-Ari *et al.*, 1997; Onimaru *et al.*, 1999). An increase in the intracellular Ca^{2+} concentration activates a wide range of intracellular cascades and is involved in neuronal growth and differentiation. Furthermore, GABA excitation and Ca^{2+} in ux may act as triggers for plasticity of synaptic connections and for establishing and patterning of neural networks. GABA-stimulated upregulation of the expression of KCC2 may be the mechanism underlying this synaptic switch (Kriegstein & Owens, 2001). This switch and expression of KCC2 can be modulated by visual experience in the retina (Sernagor *et al.*, 2003); thus both developmentally set cues and sensory experience may turn on this crucial switch from excitation to inhibition. The opposite effect – downregulation of KCC2 expression – may occur with traumatic brain injury and possibly asphyxia, inducing epileptic activity due to dysfunction of GABAergic inhibition (Rivera *et al.*, 2002). Also the subunit composition of $GABA_A$ and $GABA_B$ receptors changes during postnatal development, suggesting the existence of molecularly distinct immature and adult forms of $GABA_A$ receptors in CNS (Fritschy *et al.*, 1994; Zheng *et al.*, 1994; Benke *et al.*, 2002).

The $GABA_A$ receptors have strong a nity for benzodiazepines. Several anxiolytic and anticonvulsant drugs increase the ability for GABA to open chloride channels. In neonatal neurons, GABA currents are potentiated by barbiturates but are insensitive to benzodiazepines (Cherubini *et al.*, 1991). Considering the fundamental role of GABA in the different stages of cell development during embryonic, fetal, and postnatal life, and that it has a trophic role during early brain development, interference with the function of GABAergic transmission during this period may affect the development of neuronal wiring and the plasticity of neuronal networks and also have a profound in uence on neural organization. For a more extensive recent review of how GABAergic drugs, such as ethanol, anesthetics, and anticonvulsants, may affect brain development, see Henschel *et al.* (2008).

Ethanol, which is misused by some women during pregnancy, interacts with the $GABA_A$ receptor. The sensitive time window in rat cerebral cortex for ethanol exposure occurs between postnatal day (P)3 and P10. It is worth noting that GABA during this same period seems to have mainly depolarizing and trophic effects on developing cortical neurons through effects on cell proliferation and migration (Belhage *et al.*, 1998). In humans, the intellectual deficit produced by abnormalities of brain growth is the most important component of the fetal alcohol syndrome (FAS; Kopecky & Koren, 1998). Craniofacial abnormalities in human fetuses related to first trimester alcohol exposure are similar to the facial defects seen in $GABA_A$ subunit receptor knockout mice (Condie *et al.*, 1997). Children with FAS have often been exposed repeatedly to ethanol in utero, but it is noteworthy that research in infant rodents has demonstrated increased apoptotic neurodegeneration following brief exposures. Raising blood ethanol to 50 mg/dl (a serum concentration that may be achieved easily during social drinking) for only 30–45 minutes can be su cient to trigger a significant neuroapoptosis during synaptogenesis (Young & Olney, 2006).

Glycine

Glycine has both excitatory and inhibitory actions and can be regarded as the phylogenetically older inhibitory transmitter restricted to the brainstem and spinal cord in the adult. A similar switch as regarding the $GABA_A$ receptors from excitatory to inhibitory effects seems to occur with maturation (Miles, 1999; Gallo & Haydar, 2003). The NMDA receptor has a modulatory site where glycine in submicromolar concentrations increases the frequency of NMDA receptor channel opening. Conditions that alter the extracellular concentration of glycine can markedly alter NMDA-receptor–mediated responses (see Corsi *et al.*, 1996; Chapter 17).

The maturation of the inhibitory functions of GABAergic and glycinergic interneurons may play role in the disappearance of neonatal re exes such as grasping (Fitzgerald, 1991).

Neuropeptides

More than 50 neuropeptides have been identified. In contrast to most of the other neurotransmitters/modulators, the neuropeptides are synthesized and packaged in large dense-core vesicles in the cell soma and are carried to the nerve terminals by axonal transport at a rate of 1.5 mm/h. It is obvious that owing to this relatively slow process the neuropeptides cannot act as fast-switching neurotransmitters. Rather, they have a neuromodulatory role. They are often stored together with other neurotransmitters, i.e., monoamines or EAA, and it is possible that they play a role in setting of the sensitivity. Some of them are probably of less physiological importance and occur in the body mainly as evolutionary residues (Bowers, 1994). Still they are of great neuropharmacological interest and their analogs or antagonists can be used as drugs. The most well-known examples are the opioids and naloxone.

Opioids

Besides pain perception, endogenous opioids are involved in blood pressure and temperature regulation, feeding, sexual activity, and memory storage. Three major classes of opioid receptors, , , and κ, are currently known and have been characterized and cloned, all with putative receptor subtypes. All are seven-transmembrane proteins and members of the G-protein-coupled receptor superfamily. Endogenous opioid peptides with distinctive selectivity profiles are the enkephalin ( ), endorphin ( ), and dynorphin (κ) groups.

Neurons containing β-endorphin have long projections and primarily occur in the pituitary, whereas those containing proenkephalin- and prodynorphin-derived peptides generally have moderate to short projections (Morita, 1992). β-Endorphin exists in two main forms with different production sites and effects on the brain. The nonacetylated form is found in the anterior pituitary, is involved in fetal growth, and is expressed early during fetal brain development (E14 in the rat). The acetylated form is present in the intermediate lobe of the pituitary and is involved in postnatal development (Wang *et al.*, 1992). -Receptor binding sites are present during mid-fetal life and have a high density in cardiorespiratory-related brainstem nuclei, whereas the -opioid receptors primarily appear during the postnatal period in rats (Gaveriaux-Ruff & Kieffer, 2002).

Although opioid-binding sites progressively increase in the developing brain, the effect of opioids appears to be dependent on the status of neuronal maturation. In addition, many neuronal populations exhibit transient expression of one or the other opioid genes but the physiological role of this is not clear. Opioid agonists inhibit mitosis and DNA synthesis in

the developing brain and endogenous opioids exert potent regulatory effects on brain development and morphogenesis, as demonstrated by the administration of exogenous opioid agonist and antagonist during the fetal period (Lichtensteiger, 1998). Human neonates who have been exposed in utero to opioids such as heroin have a smaller head circumference and reduced body weight due to a decrease in cell number (Kopecky & Koren, 1998).

Substance P and other tachykinins

Substance P is a primary sensory transmitter mediating pain sensations via the thin C fibers. Substance P is also involved in the transmission of chemoreceptor and barometric input from the carotid and aortic chemo- and baroreceptors. Immunocytochemical studies have demonstrated that substance P appears in the rat brainstem at a gestational age of 14 days and reaches a maximum at a postnatal age of 21 days, and thereafter there is a successive decrease (Sakanaka, 1992). In humans there is an increase towards birth and then a leveling off during the first six months (Bergstrom *et al.*, 1984).

Substance P may play a role in neurogenesis. It seems to counteract damage induced by neurotoxins and accelerates regeneration of cortical catecholamine fibers (see Sakanaka, 1992). Increased expression of mRNA coding for pre-protachykinin A, the substance P precursor, has been recorded in respiratory-related nuclei in both the rabbit (Lagercrantz, 1996) and the rat (Wickstrom *et al.*, 1999). Increased expression of PPT-A mRNA has also been detected in patches in the caudate and putamen nuclei of the human newborn brain (Brana *et al.*, 1995). Thus, there are suggestions that substance P is involved in the resetting and adaptation of the organism to extrauterine life.

Increased levels of substance P have been found in the brainstem of infants dying of SIDS. Lower concentrations of substance P were detected in the brainstem of children dying of Rett syndrome. They were also found in reduced concentrations in the cerebrospinal uid (Matsuishi *et al.*, 1997).

NPY-related peptides

Neuropeptide Y is probably the most important of the pancreatic polypeptide family in the brain. The peptides in the family are peptide YY (PYY), avian pancreatic polypeptide (APP), and human pancreatic polypeptide (HPP). NPY is released together with norepinephrine or epinephrine (Hokfelt *et al.*, 2003; Ubink *et al.*, 2003). It is a strong vasoconstrictor and increases the sensitivity of sympathetically innervated smooth muscle. In the brain NPY has been reported to be anxiolytic and may play an important role in dampening excitotoxicity during seizures. It also has a role in the control of food intake. However, transgenic mice deficient in NPY seem to develop normally and exhibit normal food intake and body weight (Baraban *et al.*, 1997).

Galanin

Galanin is involved in cognition, nociception, feeding, and sexual behavior (Bedecs *et al.*, 1995). Of the norepinephrinergic neurons in the locus ceruleus, 80% contain galanin. Galanin hyperpolarizes these neurons and inhibits the release of norepinephrine. It can be detected at E19 in the rat fetus and is then upregulated at birth, whereas the galanin receptors seem to be downregulated (Wickström *et al.*, 1999). It may possibly modulate the effects of the norepinephrine surge at birth. Furthermore, it inhibits excessive glutamate release during perinatal asphyxia (Ubink *et al.*, 2003). NPY has recently been shown to induce neuronal precursor proliferation via Y1 receptors and also to have a trophic effect on blood vessels in the CNS (Hansel *et al.*, 2001; Hokfelt *et al.*, 2003).

Purines

Purines are not only fundamental components in the energy turnover of all cells but they also modulate neuronal activity through synaptic or nonsynaptic release and interaction with specific receptors. The purinergic receptors are divided into type 1 receptors (P1), sensitive to adenosine and AMP, and type 2 (P2), sensitive to ATP and ADP. The actions of purines are related as a rapid breakdown of ATP increases the levels of adenosine.

Purinergic mechanisms and specific receptor subtypes have been shown to be involved in various pathological conditions in the fetus, child, and adult including ischemia, brain trauma, and neurodegenerative diseases involving neuroimmune and neuroin ammatory reactions, as well as in neuropsychiatric diseases (for a recent review regarding purines, see Burnstock, 2008).

ATP

The purine nucleotide ATP is the main source of energy in cells, and is also stored in synaptic vesicles

and released together with classic transmitters such as norepinephrine and acetylcholine. The ratio between ATP and catecholamines in chroma n granules has been found to be higher during early life than later suggesting that ATP is a very early phylogenetic and ontogenetic signaling substance (O'Brien *et al.*, 1972). During the past decades, evidence for ATP as a neural signaling substance has emerged by examining sites of storage, release, and hydrolysis, as well as potential actions and targets. A variety of receptors for extracellular ATP have been identified. Some are involved in fast neuronal transmission and operate as ligand-gated ion channels ($P_{2T,\ X,\ and\ Z}$). Others are involved in the paracrine or autocrine modulation of cell function ($P_{2U\ and\ Y}$). Many receptors of this type are coupled to phosphoinositide-specific phospholipase C (Fredholm, 1997). Intracellular ATP levels directly change the excitability of neurons by ATP-dependent potassium channels which may hyperpolarize cells, thus decreasing neuronal activity when energy resources are scarce. Moreover, the release of ATP through unopposed gap junction channels, "connexins" (Elias & Kriegstein, 2008), or "pannexins" (Iglesias *et al.*, 2009), as well as intercellular ATP signaling are essential for the migration of neural progenitor cells and the proper formation of the subventricular zone. Interference with ATP signaling or abnormal calcium uctuations in basal or intermediate neuronal progenitors may play a significant role in a variety of genetic and acquired cortical malformations (Liu *et al.*, 2008).

Adenosine

Adenosine is a constituent of all body uids, including the extracellular space of the CNS. It has multiple effects on organs and cells of the body. Thus, its levels are tightly regulated by a series of enzymatic steps (Fredholm, 1997). Adenosine can be regarded more as a neuromodulator, in that it does not seem to be stored in vesicles. Adenosine is produced by dephosphorylation of adenosine monophosphate (AMP) by 5′-nucleotidase, an enzyme occurring in both membrane-bound and cytosolic forms. Degradation of intra- and extracellular ATP is the main source of extracellular adenosine. Specific bidirectional transporters maintain intra- and extracellular concentrations of adenosine at similar levels. During basal conditions adenosine levels are 30–300 nM and can rise following stimuli that cause an imbalance between ATP synthesis and ATP breakdown. Thus, the levels during ischemia or hypoxia can rise 100-fold (Winn *et al.*, 1981b; Fredholm, 1997). The extracellular concentrations of adenosine might be higher in the fetal brain than postnatally, since fetal PaO_2 can decrease below the level (30 mmHg) when a marked increase in extracellular adenosine can be expected (Winn *et al.*, 1981a). Overall, adenosine decreases oxygen consumption and has neuroprotective effects (Arslan *et al.*, 1997). However, hypoxia also induces a decrease in neonatal respiration. Theophylline and caffeine are adenosine antagonists that cause ventilation to increase and that decrease the incidence of neonatal apneas when given systemically, mainly due to the antagonistic effect of theophylline on adenosine A_1 receptors in the medulla oblongata (Herlenius & Lagercrantz, 1999). A variety of receptors for extracellular ATP have been identified. Specific adenosine receptors interact with G-proteins, adenosine A_1, expressed pre- and post-synaptically in neurons ubiquitously, highest in hippocampus, and A_3 receptors mainly interact with G(i/o) proteins. A_{2a} and A_{2b} receptors mainly interact with G(s) proteins. A_{2a} receptors are enriched in basal ganglia and are closely associated and functionally interact with dopamine D_2 receptors (Fredholm *et al.*, 2001; Stevens *et al.*, 2002; Fredholm & Svenningsson, 2003). Oligodendrocyte progenitor cells (OPCs) express functional adenosine receptors, which are activated in response to action potential firing. Adenosine acts as a potent neuron–glial transmitter to inhibit OPC proliferation, stimulate their differentiation, and promote the formation of myelin (Stevens *et al.*, 2002).

The general level of neuronal activity and metabolic processes that support it may be unusually high in the human cortex, and upregulation of several genes involved in synaptic transmission is a characteristic of the human compared with non-human primate brain (Caceres *et al.*, 2003). The metabolic control of brain activity by adenosine thus could be even more important in humans than in other mammals. Some caution against extensive use of adenosine receptor antagonists such as caffeine has been recommended in pregnant women and preterm infants (Schmidt, 1999; Herlenius *et al.*, 2002). On the other hand, it has recently been demonstrated that infants who have been treated with caffeine show improved neonatal outcome as compared with controls (Schmidt *et al.*, 2006, 2007).

Perinatal transition

Before birth

The levels of most neurotransmitters and neuromodulators increase concomitantly with synapse formation. Some of them surge during the perinatal period, such as glutamate, catecholamines, and some neuropeptides, and then level off. The interesting question is to what extent the expression of neuroactive agents is related to the functional state of the fetus and the newborn. On the one hand, there is an intense firing and wiring in the fetal brain, particularly during active sleep. The inhibitory neurotransmitter GABA is mainly excitatory in the fetal period (see above). Amino acid transmitters also act via NMDA receptors, which are important for the wiring and plasticity of the immature brain, although the main excitatory fast-switching receptors (AMPA) are expressed later. On the other hand, activities such as respiratory movements are suppressed. The fetus seldom or never becomes aroused or wakes up. The sympathetic tone is low. Furthermore, the fetus is adapted to the low oxygen level in the womb – "Mt. Everest in utero." If a fetus is challenged by asphyxia it is not excited as an adult responding with a ight or fight reaction, but rather it becomes immobilized, stops breathing, and becomes bradycardic (see Lagercrantz, 1996). This paralytic state of the fetus can be caused by inhibition of the chemical neurotransmission. Adenosine is such a neuromodulator that might be involved in this suppression of the fetal brain. It has a general sedative effect. Its concentration increases during energy failure and hypoxia, and it has been suggested that it can act as a modulator to cope with the hypoxic situation (Berne, 1986). Adenosine A_1 receptor activation depresses breathing substantially in the fetus and the neonate by inhibiting synaptic transmission and hyperpolarizing certain neurons (Herlenius & Lagercrantz, 1999). PGE_2, released from the placenta, also contributes to the inhibition of the fetus, and although the decrease of this placental inhibitor is not crucial for establishing continuous breathing movements at birth, its removal allows the newborn baby to be vigilant after birth (Alvaro *et al.*, 2004). In addition, release of PGE_2 and inhibition of the brain activity at birth also occur by hypoxia-induced activation of brain microsomal prostaglandin synthase-1 (mPGES-1) and subsequent release of endogenous PGE_2 (Hofstetter *et al.*, 2007).

Inhibition of fetal activity may also be mediated by the maternal oxytocin released in high concentrations during labor. Oxytocin has been found to reduce the intracellular concentration of chloride and thus switch the effect of GABA from an excitatory to an inhibitory neurotransmitter (Tyzio *et al.*, 2006).

Thus, high levels of adenosine and prostaglandins together with the birth-related switch of GABA into an inhibitory neurotransmitter all contribute to allow the stressful birth to be endured without damage to the temporarily inhibited, and thus less-energy-consuming, brain.

Neuropeptides that might be involved in the suppression of fetal activity are NPY, somatostatin, and endogenous opioids. The levels of NPY are relatively high in the fetal brain and decline after birth. Plasma levels of endorphins and enkephalins are increased in the umbilical cord at birth (Ramanathan *et al.*, 1989; Aurich *et al.*, 1990).

Birth

The healthy newborn baby is aroused and awake the first two hours after birth and starts continuous breathing movements. Factors such as squeezing and squashing of the fetus, increased sensory input, and cooling are probably important. We can hypothesize that there is a surge of excitatory neurotransmitters and downregulation of inhibitory ones in the brain.

The increased neuronal activity is indicated by the increased expression of immediate early genes (Ringstedt *et al.*, 1995). The arousal and vigilance of the newborn seem to be related to activation of the norepinephrinergic system in the brain, particularly the locus ceruleus, from where norepinephrinergic neurons are distributed in the whole brain (see above). The norepinephrine turnover as indicated by the ratio of the metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) to norepinephrine was increased twofold to threefold in the newborn rat (Lagercrantz, 1996). There are indirect indications that there is also a norepinephrine surge in the human brain, by the finding of high level of plasma catecholamines after birth.

A rapid decrease of the inhibitory neuromodulator adenosine in the brain occurs as partial pressure of oxygen in arterial blood rapidly increases after birth, probably contributing to the increased activity in the newborn infant compared with the fetus. In addition, a decreased sensitivity during the first postnatal days for adenosine seems to contribute to the maintenance of

continuous breathing (Herlenius & Lagercrantz, 1999; Herlenius *et al.*, 2002).

Prenatal and perinatal programming

The concept of fetal and neonatal programming discovered by Barker (Sayer *et al.*, 1997) also applies to the ontogeny of neurotransmitters and neuromodulators, i.e., an early stimulus or insult at a critical period can result in long-term changes in the structure and the function of the organism (see Chapter 22). For example, it can be postulated that prenatal or perinatal stress can disturb the timetable of the expression of neurotransmitters and neuromodulators and their receptors. Disruption of the normal timing or intensity of neurotransmitter signaling can lead to permanent changes in proliferation, differentiation, and growth of their target cells during critical phases of development of the nervous system, thereby possibly providing the underlying mechanisms for neurobehavioral or neurophysiological abnormalities associated with developmental exposure to neuroactive drugs and environmental toxins.

Hydrocortisone given to neonatal rats has been found to enhance the maturation of the monoaminergic systems in the brain (Kurosawa *et al.*, 1980). Administration of extra glucocorticosteroids to the rat fetus induces alterations of dopamine receptor responses, which affects the spontaneous motor control both in short- and long-term perspectives (Diaz *et al.*, 1997). Chronic high endogenous corticosteroid levels can be induced by stress to the mother before birth, or to the child after birth. Exogenous corticosteroids are also administered by physicians to the growing infant (and brain) in the management of a wide spectrum of pre- and postnatal conditions. The long-term effects of corticosteroids on the developing human CNS as well as the long-lasting effects are not well known. However, corticosteroids have been shown to have deleterious effects on the developing brain and behavior in several animals including primates, i.e., inhibition of neural stem cells, neurogenesis, and migration leading to irreversible decrease in brain weight (Edwards & Burnham, 2001; Matthews, 2001).

Chronic prenatal hypoxia alters the monoamine turnover in the locus ceruleus and nucleus tractus solitarius in the adolescent rat (Peyronnet *et al.*, 2002). This was related to disturbed control of respiratory behavior. Human handling of newborn rats for 15 minutes during the first weeks of life affects ascending serotonergic projections into the hippocampus and causes a long-lasting increase in glucocorticoid receptors (Sapolsky, 1997).

There are also clinical studies indicating that prenatal stress is associated with attention deficit disorders in children (Weinstock, 1997). Birth insult and stress alter dopamine transporter binding in rat, possibly also leading to hyper-locomotion (El-Khodor & Boksa, 2002). People with schizophrenia seem to have experienced more pregnancy and birth complications than their healthy siblings (Stefan & Murray, 1997). For example mothers of schizophrenic patients more often had severe infections during pregnancy, possible affecting cytokines (such as IL1β, IL6, and TNFα) and indirectly the development of monoaminergic circuits in the fetal brain (Gilmore & Jarskog, 1997; Jarskog *et al.*, 1997; Meyer & Feldon, 2009).

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Historic box 4 Soups and sparks: on the history of nervous communication
Hugo Lagercrantz

In 1877, the German physiologist Emil Du Bois-Reymond wrote that there were only two ways a nerve can cause muscle contraction, either by a chemical substance or electrically. This was the beginning of a longlasting debate on chemical versus electrical neurotransmission – "The war of the soups and the sparks" (Valenstein, 2005).

Thomas Elliott is usually credited with the discovery of chemical neurotransmission. As a student he hypothesized that sympathetic nerves secrete adrenaline (epinephrine) when innervating visceral organs (1905). Charles Sherrington coined the term synapse (1897) based on Ramon y Cajal's neuron theory. He used the Greek word synapse meaning "to clasp" to refer to the junction point between neurons. Sherrington was an electrophysiologist favoring the electrical theory. John Eccles was his prominent pupil and originally the strongest opponent of chemical transmission. Henry Dale, who discovered the bradycardic effects of acetylcholine, became the leader of the "soup team."

Otto Loewi did the crucial experiment demonstrating chemical neurotransmission in 1920 in Graz, Austria. According to Loewi, the idea came up in a dream one night. However, he forgot the dream but it came back the following night. He immediately woke up and scribbled down the dream and went to the laboratory and performed the experiment. By transferring the fluid of a stimulated heart preparation from a frog onto another isolated frog heart, he found that the second heart's rate slowed down when it was perfused with the fluid from the first heart. This demonstrated that a chemical had been released from the nerve endings of the vagus – "vagusstoff," which was found to be identical with acetylcholine.

However, it took a long time before John Eccles and other electrophysiologists accepted the idea of chemical transmission. Eccles argued vigorously in stand-up fights against Henry Dale at the Physiological Society. It is said that Eccles changed his mind first when one of his collaborators in Sydney cut his lawn with an electric lawnmower. Unfortunately, the young guest scientist (Bernard Katz) succeeded in cutting the electric wire to the lawnmower, which is why Eccles decided to buy a lawnmower based on chemical transmission, i.e., a petrol-driven one.

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Chapter 8

Glial cell biology

Arne Schousboe and Helle S. Waagepetersen

Neuronal–glial interaction in nutrition and amino-acid-mediated neurotransmission

Nutrition

The main substrate for brain energy metabolism is glucose, and in the adult human brain the glucose consumption, termed cerebral metabolic rate for glucose (CMRgluc), is around 20 μmol/h per g wet weight (Sokoloff, 1960). Alternative substrates may, however, be used and in this regard ketone bodies may have an important role particularly in the infant brain. The unique anatomical location of astrocytes, that is with their end-feet closely apposed to the capillaries (Fig. 8.1), has led to the proposal (Pellerin & Magistretti, 1994; Magistretti & Pellerin, 1996) that astrocytes may be the major site for uptake of glucose in the brain. As glucose will be rapidly converted into glucose 6-phosphate, which cannot cross the cell membrane, it is likely that metabolism of glucose proceeds to form lactate, which may subsequently be transferred from the astrocytes to the neurons via the monocarboxylic acid transporters present in the membranes of both types of brain cell (Fig. 8.1) and possibly most prevalent in neurons (Pellerin *et al.*, 1998). The lactate concentration is augmented in the brain subsequent to stimulation; however, evidence for a subsequent net oxidation in the adjacent neurons is lacking (Hertz *et al.*, 2007). Interestingly, as determined by microdialysis, astrocytes and neurons oxidize 50% each of the interstitial lactate in freely moving rats (Zielke *et al.*, 2007). In this context, it should be mentioned that stimulation of glutamatergic activity in cultured neurons seems to evoke metabolism of glucose to a larger extent than that of lactate (Bak *et al.*, 2006). The possibility that neurons can utilize lactate as an energy substrate is important under conditions of short-lasting failures in the glucose supply, as this allows the use of astrocytic glycogen stores as an emergency fuel source in neurons. This is based on mobilization of glycogen by conversion to glucose 1-phosphate that, via glycolysis, is metabolized to lactate, which is subsequently transferred to neurons. It has been demonstrated that stimulation of glycogen metabolism in astrocytes by activation of β-epinephrinergic receptors leads to production and release of lactate (Dringen *et al.*, 1993). These aspects are illustrated in Fig. 8.1, which schematically shows the metabolic interactions between neurons and astrocytes with regard to exchange and transfer of metabolites. The role of glycogen in relation to the maintenance of neurotransmission processes is currently being investigated. Such studies have been facilitated by the availability of specific inhibitors of glycogen phosphorylase, the enzyme responsible for production of glucose 1-phosphate (see above). Several studies have provided evidence for an active role of glycogen in this context and this is often referred to as the glycogen shunt, i.e., flux of glucose through glycogen prior to entering the glycolytic pathway as shown in Fig. 8.1 (Shulman *et al.*, 2001; Brown *et al.*, 2005; Sickmann *et al.*, 2005; Dienel *et al.*, 2007).

Amino acid transmission

Neurotransmission mediated by the excitatory transmitter glutamate and the inhibitory neurotransmitter γ-aminobutyrate (GABA) obviously requires release of these amino acids from the nerve endings (Fig. 8.1). The loss of glutamate and GABA from glutamatergic and GABAergic neurons, respectively, is, to some extent, compensated for by reuptake of these amino acids (Schousboe, 1981; Hertz &

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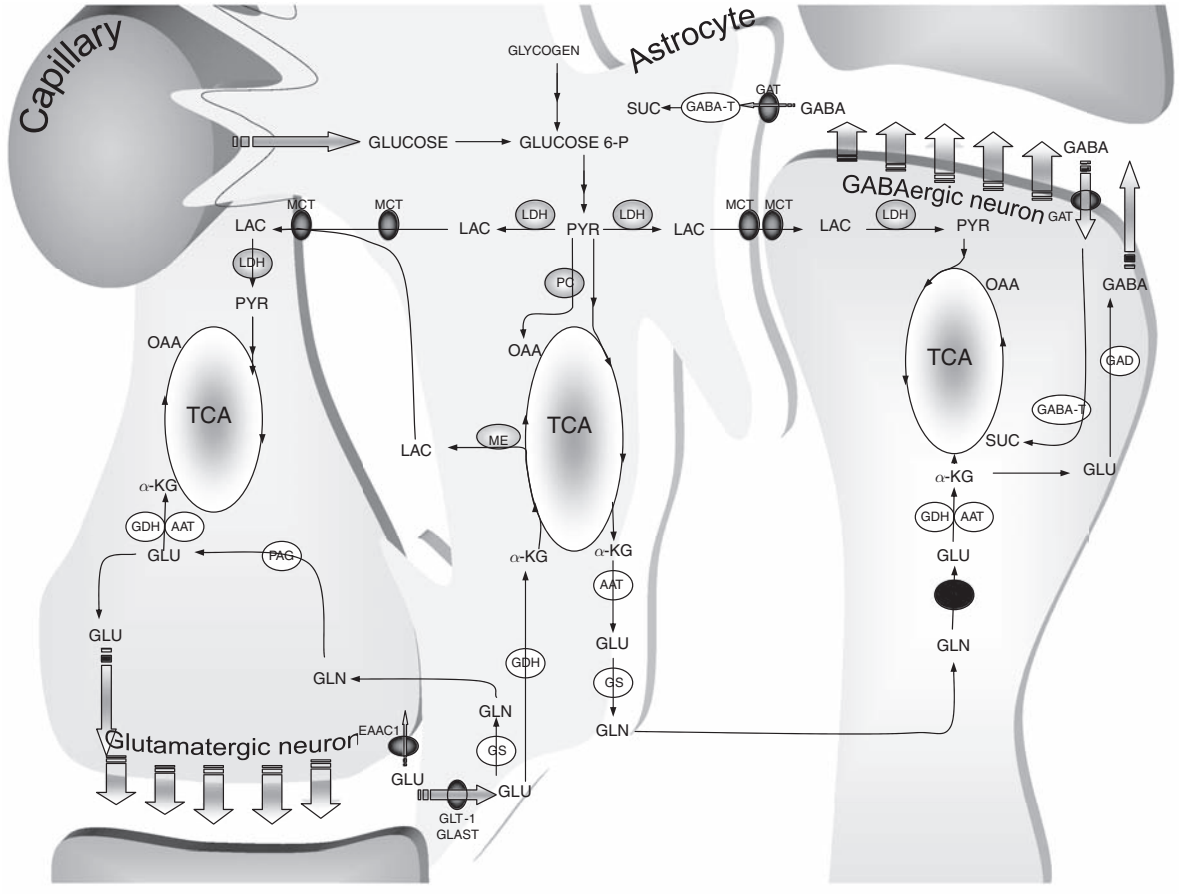

Fig. 8.1 Schematic representation of a microenvironment in the brain consisting of a capillary, an astrocyte, and a glutamatergic and a GABAergic neuron. Release and uptake processes for the neurotransmitter amino acids glutamate (GLU) and GABA are indicated by bold arrows, the size of which semi-quantitatively reflects the corresponding activities. The membrane transporters for glutamate are named EAAC1 (neuronal) and GLT-1/GLAST (astrocytes) and those for GABA are called GAT. The lactate transporters are named MCT. It should be noted that succinate produced from GABA feeds into the tricarboxylic acid (TCA) cycle. Enzymes are abbreviated as follows: GDH, glutamate dehydrogenase; PAG, phosphate activated glutaminase; AAT, aspartate aminotransferase; GS, glutamine synthetase; GAD, glutamate decarboxylase; GABA-T, GABA-transaminase; ME, malic enzyme; PC, pyruvate carboxylase; LDH, lactate dehydrogenase. Metabolites are abbreviated as follows: LAC, lactate; PYR, pyruvate; OAA, oxaloacetate; α-KG, α-ketoglutarate; SUC, succinate, GLN, glutamine

Schousboe, 1987; Schousboe *et al.*, 1988). However, for both amino acids there will be a net loss from the neurons, a loss which is most pronounced in the case of glutamate. This requires compensatory mechanisms allowing the neurons to perform de novo synthesis of the amino acids. It was originally proposed that glutamine, which is synthesized exclusively in astrocytes by glutamine synthetase (Norenberg & Martinez-Hernandez, 1979), could function as a substrate for biosynthesis of the amino acids thus stoichiometrically compensating for the loss of glutamate and GABA (see Westergaard *et al.*, 1995). On the basis of subsequent demonstration that glutamate metabolism in astrocytes proceeds to glutamine and also to a large extent involves oxidative metabolism to carbon dioxide and/or lactate (Yu *et al.*, 1982; Sonnewald *et al.*, 1993, 1997; McKenna *et al.*, 1996), it is unlikely that the transfer of glutamine from astrocytes to neurons can occur in a stoichiometric manner, as discussed in detail by Westergaard *et al.* (1995). The fact that neurons lack not only glutamine synthetase (see above) but also the main anaplerotic enzyme in the brain, pyruvate carboxylase (Yu *et al.*, 1983; Shank *et al.*, 1985; Cesar & Hamprecht, 1995), makes it essential that tricarboxylic acid (TCA) cycle constituents can be replenished in neurons by transfer from

astrocytes. As discussed by Westergaard and colleagues (1995), such compensatory transfer does occur to some extent. It thus appears that glutamine, together with TCA cycle constituents, represents the main source of precursors for biosynthesis of glutamate and GABA in neurons. It should be emphasized, however, that in quantitative terms the flux of glutamine between astroglia and neurons is more important than transfer of TCA cycle constituents (Westergaard *et al.*, 1995). It may also be noted that recently it was demonstrated that presynaptic uptake of glutamate into glutamatergic neurons plays a role in the maintenance of the transmitter pool (Waagepetersen *et al.*, 2005).

Developmental aspects of glial cell biochemistry

Enzymes

One of the important functions of astrocytes is to supply the metabolically handicapped neurons with energy substrates as well as precursors for synthesis of neuroactive amino acids (see Hertz *et al.*, 1992). This means that astrocytes during the developmental period of the brain acquire high levels of activities of key enzymes such as lactate dehydrogenase, pyruvate carboxylase, malic enzyme, glutamate dehydrogenase, aspartate aminotransferase, glutamine synthetase, and GABA-transaminase (Schousboe *et al.*, 1977a, b; Hertz *et al.*, 1978; Yu *et al.*, 1983; Kurz *et al.*, 1993), and the astrocyte-specific enzymes pyruvate carboxylase and glutamine synthetase exhibit almost identical developmental profiles in the brain in vivo in mice and in mouse neonatal astrocytes in culture (Hertz *et al.*, 1978; Yu *et al.*, 1983). It should, however, be noted that studies of the activity of glutamine synthetase in the intact tissue or neuronal–astrocytic co-cultures indicate that the functional activity is influenced by neuronal factors (Wu *et al.*, 1988).

One of the most important functions of the astrocytes surrounding glutamatergic nerve terminals is to keep the concentration of glutamate in the synaptic cleft at a level below that which may induce excitotoxic damage to neurons (see Choi, 1988; Schousboe & Frandsen, 1995; Schousboe & Waagepetersen, 2005). It would therefore be expected that the enzymatic machinery for glutamate metabolism is present in astrocytes at a high expression level. As mentioned above, this is the case for glutamine synthetase, which is only expressed in astrocytes. From studies on the activities of glutamate dehydrogenase (GDH) in neurons and astrocytes and from immunocytochemical labeling of the enzyme in brain slices (Schousboe *et al.*, 1977b; Drejer *et al.*, 1985; Larsson *et al.*, 1985; Rothe *et al.*, 1994), it is clear that this enzyme is expressed in astrocytes at a high level. Functional studies in intact astrocytes have shown that GDH primarily catalyzes oxidative deamination of glutamate, a process that appears to be very important for metabolic degradation of glutamate to α-ketoglutarate, which can subsequently be oxidized in the TCA cycle (see Sonnewald *et al.*, 1997). Alternatively, glutamate could be transaminated to α-ketoglutarate via aspartate aminotransferase which also has high activity in astrocytes. It appears, however, that this enzyme preferentially catalyzes production of glutamate from α-ketoglutarate (Westergaard *et al.*, 1996). It should be noted that oxidation of glutamate in the TCA cycle leads to production of lactate, a process that probably involves malic enzyme (Sonnewald *et al.*, 1993). This lactate will subsequently be available for neurons that can utilize lactate as a supplementary energy substrate (Magistretti & Pellerin, 1996; Waagepetersen *et al.*, 1998a, b).

It has been shown by nuclear magnetic resonance (NMR) analysis that metabolism of [U-^{13}C]glutamate in astrocytes via GDH is particularly pronounced at high extracellular glutamate concentrations (McKenna *et al.*, 1996; Sonnewald *et al.*, 1997). This may be explained by the fact that GDH is allosterically activated by adenosine diphosphate (ADP) (McCarthy & Tipton, 1983), the concentration of which is likely to increase when glutamate uptake is intense. This is because it is a process requiring energy in the form of adenosine triphosphate (ATP), which is converted to ADP.

In addition to being able to metabolize glutamate, astrocytes are equipped to metabolize the inhibitory neurotransmitter GABA, because the GABA-metabolizing enzyme GABA-transaminase is present in astrocytes at high activity (Schousboe *et al.*, 1977a). The carbon skeleton of GABA subsequently enters the TCA cycle in the form of succinate (Fig. 8.1). GABA, which is metabolized in astrocytes or in neurons, is lost from the neurotransmitter pool, and this appears to be important for optimal function of inhibitory neurotransmission. Thus, drugs that block GABA metabolism, e.g., vigabatrin and valproate, act as anticonvulsants by increasing the availability of GABA in the neurotransmitter pool (Schousboe, 1990; Waagepetersen *et al.*, 1999).

Glutamate transporters

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS), acting on a variety

of receptors coupled to ion channels or G-proteins and second messenger systems (Schoepp & Conn, 1993; Lodge, 1997). The extracellular concentration of glutamate is kept at a very low level by the highly efficient glutamate transporters in the membranes of neurons and glial cells (Nicholls & Attwell, 1990). From studies of glutamate transport in neural cellular and subcellular preparations, it has become clear that glutamate uptake into glial elements acts as the quantitatively most important mechanism for removal of glutamate from the extracellular space as indicated in Fig. 8.1 (Hertz, 1979; Schousboe, 1981; Lehre & Danbolt, 1998). The recent cloning of a series of glutamate transporters from the CNS and subsequent studies of their cellular localization have confirmed this notion (Danbolt, 1994; Gegelashvili & Schousboe, 1997; Danbolt, 2001).

Uptake of glutamate increases as a function of postnatal development in the brain (Schousboe *et al.*, 1976), and in astrocytes in culture the capacity for glutamate uptake is dependent on the stage of maturation and differentiation of the cells (Hertz *et al.*, 1978; Gegelashvili & Schousboe, 1997). This is in all likelihood related to the fact that a variety of environmental cues such as factors released from neurons are known to enhance expression of glutamate transporters in astrocytes (Drejer *et al.*, 1983; Gegelashvili *et al.*, 1996, 1997; Gegelashvili & Schousboe, 1997). This probably explains the observation that destruction of glutamatergic neuronal pathways leads to a reduction of glutamate uptake in the brain areas in question (Levy *et al.*, 1995).

Since glutamate uptake requires an intact sodium gradient and membrane potential in order to function optimally (Nicholls & Attwell, 1990), it is clear that conditions such as energy failure will affect the ability of cells to maintain the very high intra/extracellular glutamate gradient present under physiological conditions. This failure of glutamate uptake will reverse the direction of the carriers resulting in the marked increase in the extracellular glutamate concentration seen during ischemia (Benveniste *et al.*, 1984; Levy *et al.*, 1998). This is a major contributing factor in the neuronal degeneration associated with ischemia because glutamate acts as a very potent neurotoxin (Schousboe & Frandsen, 1995; Schousboe & Waagepetersen, 2005). In relation to this, it should be noted that other neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS) have been associated with a reduction in the expression of GLT-1, the most important glial glutamate transporter (Rothstein *et al.*, 1992, 1993). As pointed out above, failures in glutamate metabolism may also have important roles in this context (Plaitakis, 1990).

GABA transporters

GABA, which is produced from glutamate by decarboxylation catalyzed by glutamate decarboxylase (Schousboe, 1981; Waagepetersen *et al.*, 1999), acts as the major inhibitory neurotransmitter in the CNS (Roberts, 1991). Its inactivation as a transmitter is mediated by high-affinity transporters residing in GABAergic neurons and surrounding astrocytes (Schousboe, 1981). Contrary to glutamate, the highest capacity for GABA uptake appears to be associated with GABAergic nerve endings (Hertz & Schousboe, 1987). This may be compatible with the view that GABA neurotransmission to a large extent is based on reutilization of released transmitter GABA (Fig. 8.1). Thus, in GABAergic neurons, exogenously supplied GABA is accumulated into the vesicular neurotransmitter pool (Gram *et al.*, 1988; Schousboe, 1990).

High-affinity GABA transport is also expressed in astrocytes (Schousboe *et al.*, 1977a) although, as mentioned above, the capacity for uptake seems lower than that present in GABAergic neurons (Hertz & Schousboe, 1987). Like GABA uptake in the brain, the astrocytic uptake process is developmentally regulated, being increased in the course of postnatal development (Schousboe, 1981). Like most other uptake systems for amino acids, GABA uptake is Na^+ dependent, which makes the uptake electrogenic and thus dependent on the membrane potential (Martin, 1976; Schousboe, 1981). In this context, it is of functional importance that, in astrocytes, the coupling ratio between Na^+ and GABA (Na^+: GABA ratio) increases as a function of development (Larsson & Schousboe, 1981), a property that is shared by the neuronal GABA uptake (Larsson *et al.*, 1983). As a consequence of this, the efficiency of GABA uptake increases in the brain during postnatal development. This may be of importance in the light of the fact that GABA appears to have an important role as a neurodifferentiating factor during early development (Meier *et al.*, 1991). In order for GABA to fulfill this function, the extracellular concentration needs to be kept at a relatively high level compared with that seen in the adult brain, a condition that would be incompatible with a highly efficient uptake system, since GABA synthesis and release are limited

during the early developmental period (Waagepetersen *et al.*, 1999).

Also of interest is that astroglial GABA uptake may be influenced by neuronal stimuli. This was originally demonstrated in cultured cerebellar astrocytes where the expression of GABA transporters could be enhanced by conditioned media taken from cultured neurons (Drejer *et al.*, 1983). It was subsequently shown that the active component in these media is a glycoprotein with a molecular weight of approximately 30 kDa (Nissen *et al.*, 1992). Although the mechanism of action of this protein, termed GABACIP, has not been fully characterized, it is clear that it acts via stimulation of de novo synthesis of GABA carriers in astrocytes. In the light of the recent cloning of four different mouse brain GABA carriers (GAT1–4), which exhibit different pharmacological and functional characteristics (Borden, 1996; Bolvig *et al.*, 1999; Clausen *et al.*, 2006; Madsen *et al.*, 2008), it would be of interest to study the action of this neuronal factor on expression of these transporters in astrocytes. This may be particularly relevant since it has been recently shown that the GABA transporter GAT2, which is also known as the betaine transporter (BGT-1), plays a prominent role together with GAT1 in the control of seizure or epilepsy-like activity (Schousboe *et al.*, 2004; White *et al.*, 2005; Clausen *et al.*, 2006).

Concluding remarks

Since 1980 the understanding of the functional importance of astrocytes in the brain has changed from that of a static role as a mechanical support for neurons to that of a highly dynamic one where a constant interplay between neurons and astrocytes exchanging metabolites and neurotransmitters appears to be a prerequisite for normal brain function. The present chapter has concentrated on a discussion of nutritional and amino acid homeostatic functions of astrocytes. It should be emphasized, however, that astrocytes are involved in many other basic brain functions, such as ion homeostasis, cell volume regulation, and regulation of monoamine neurotransmission (Kettenmann & Ransom, 1995). Astrocytes therefore need to be considered as very active participants in essentially all brain functions, and it should be noted that they express a large repertoire of neurotransmitter receptors, which allows constant monitoring of neuronal activity (Kettenmann & Ransom, 1995). Proper development of astrocytes during embryogenesis and the neonatal period therefore is of utmost importance for proper function of the CNS in adulthood. It may be important for better understanding of astrocytic function in the brain that it has recently been possible to delineate the enzymatic and metabolic machinery in a transcriptomic analysis of acutely isolated protoplasmic astrocytes from mouse brain (Lovatt *et al.*, 2007).

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Section 2
Chapter 9

Sensory systems and behavior

Development of the somatosensory system

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Introduction

The somatosensory system deals with information from a variety of sensory receptors located in the skin, muscles, joints, and other deeper tissues. It enables us to experience touch, pain, warmth, and cold, and to sense the position and movements of our body. Understanding how this system develops structurally and functionally during embryonic and fetal life provides an insight into how the fetus and newborn infant develops the capacity to receive and experience sensations arising from noxious, tactile, thermal, or mechanical stimuli. Although it will be difficult to determine, unequivocally, when the fetus is first aware of its surroundings and conscious of perceiving these stimuli, we can at least define when the minimum structural and functional apparatus necessary to do so is present.

In this chapter we will first describe the structure of the main pathways that transmit tactile, thermal, nociceptive, and proprioceptive information from the periphery to the cerebral cortex via synaptic connections in the spinal cord or brainstem. We will then describe the structural, neurochemical, and functional development of the somatosensory system and the development of descending pathways from the brainstem which modify this activity. We will speculate on whether activity in the fetal somatosensory pathways is a necessary requirement for the appropriate development of these pathways as it appears to be for the visual system (Goodman & Shatz, 1993; Penn & Shatz, 1999). Clearly, very little experimentation can be performed on the human fetus, and laboratory animals are therefore used to answer these questions. We will draw on data mainly from the fetal rat (gestation ~21.5 days) and fetal sheep (gestation 21 weeks). A considerable proportion of the development of the central nervous system (CNS) in sheep occurs in utero as it does in the human, making sheep a particularly useful animal model for the human fetus. Where available, data from humans are also included.

Somatosensory receptors and pathways

Information from receptors in the body reaches the cerebral cortex via two main ascending systems: the dorsal column–medial lemniscal system and the spinothalamic or anterolateral system. The principal anatomical features of these systems are outlined in Fig. 9.1. The receptors that feed into the dorsal column–medial lemniscal system are encapsulated, low-threshold, cutaneous mechanoreceptors and muscle spindle and tendon organ receptors. Low-threshold mechanoreceptors – specifically Meissner's, Merkel's, Ruffini's, and Pacinian receptors – in glabrous or smooth skin are innervated by large myelinated fibers of the Aβ group in the peripheral nerve. Each nerve ending responds to gentle mechanical stimulation of an area of skin that forms the receptive field. Afferents from Merkel's and Ruffini's endings have a static response – actions potentials are elicited as long as deformation of the receptive field is maintained. Ruffini's endings are particularly sensitive to lateral stretch of the skin. Fibers from Merkel's endings are known as slowly adapting type 1 fibers; those from Ruffini's endings as slowly adapting type 2 fibers. Afferents from Meissner's and Pacinian endings (which are encapsulated and have a lamellar, or layered, structure) have a dynamic response – action potentials are elicited only when deformation of the receptive field is changing. Meissner's afferents (rapidly adapting type 1) respond preferentially to stimuli that are changing relatively slowly while Pacinian afferents (rapidly adapting type 2) are most sensitive to rapidly changing stimuli. In hairy skin, there are two further classes of afferents, both of

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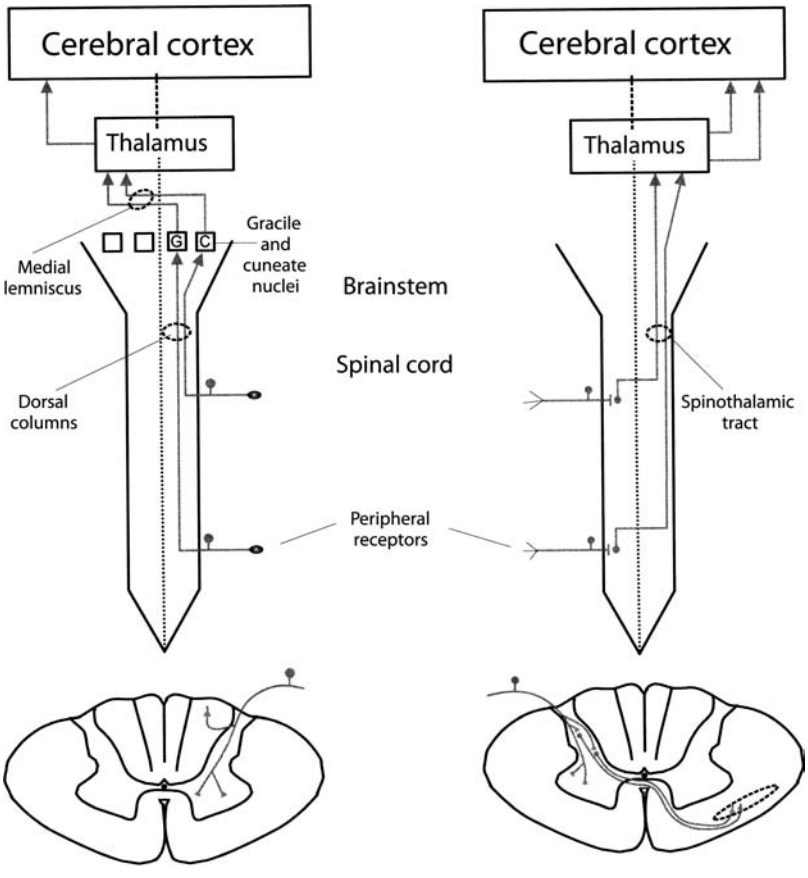

Fig. 9.1 The main somatosensory pathways from the body.

Left: Dorsal column–medial lemniscal systems. Large-diameter myelinated fibers are connected with cutaneous mechanoreceptors, and muscle spindle and tendon organ receptors provide collaterals or branches that ascend in the dorsal columns of the spinal cord and synapse in the dorsal column nuclei in the medulla – the gracile nucleus for the lower limbs and lower body and the cuneate nucleus for the upper limbs and upper body. From here, the input is transmitted via the medial lemniscus to the contralateral thalamus (ventral posterior lateral nucleus). This input system terminates in the primary somatosensory cortex and is responsible for discriminative tactile sensation, kinesthesia, and probably also for a sense of muscle tension. Although proprioceptive input from the upper limbs travels in the cuneate fasciculus to the cuneate nucleus, information from muscle spindles and Golgi tendon organs in hindlimb muscles follows a different route to the brainstem. Second-order fibers ascend in the dorsal spinocerebellar tract in the dorsolateral funiculus of the spinal cord. Tract fibers provide branches to nucleus Z in the medulla which then sends a projection to the contralateral medial lemniscus. This unusual route may help to explain why joint position sense from the lower limb may be present after dorsal column lesions.

Right: Anterolateral or spinothalamic systems. Unmyelinated and small-diameter myelinated fibers connected with nociceptors, thermoreceptors, and unencapsulated tactile receptors project into the dorsal horn. The C-fiber afferents terminate in layer II (substantia gelatinosa) of the dorsal horn of the spinal cord (see Willis & Coggeshall, 2004), and A fibers in layers I and V (see Willis & Coggeshall, 2004). Large, myelinated cutaneous afferents innervating low-threshold mechanoreceptors project to layers III–V (see Willis & Coggeshall, 2004). Axons arising from layers I, II, and V project directly to the thalamus (ventral posterior, ventromedial pars oralis, posterior, and intralaminar nuclei) via the contralateral spinothalamic tract. Recipient thalamic cells project to the primary somatosensory cortex, the limbic cortex, and the insula. A small number of touch and position sense fibers also travel in the spinothalamic system.

which have large receptive fields and both of which are rapidly adapting. All of these cutaneous fibers provide information that is experienced consciously as exquisitely subtle qualities associated with touch and texture.

Muscle spindles provide precise signals about the lengths of muscles and the velocities of stretch and hence about the position and movement of attached muscles. Spindles are composed of specialized muscle fibers, the intrafusal fibers, enclosed in a uid-filled, fusiform, fibrous capsule. Motor nerve fibers (efferent fibers) innervate the intrafusal fibers and adjust their lengths according to the state of stretch of the muscle. This is detected by the primary (Aα) afferent fibers, which make spiral terminations around the intrafusal fibers. Golgi tendon organs are encapsulated

nerve endings located at the musclo-tendinous junctions, and signal tension within the muscle, which may translate to the force of muscle contraction (if the muscle is shortening) or to load on the limbs. There are receptors in joint capsules that signal joint angle, but they are relatively sparse and may respond only at extremes of joint angle.

The large-diameter myelinated nerve fibers connected with the above-mentioned receptors provide collaterals or branches that ascend in the dorsal columns of the spinal cord and synapse in the dorsal column nuclei in the medulla. From these nuclei, fibers project via the medial lemniscus to the contralateral thalamus and then to the somatosensory cortex (Fig. 9.1); the spinothalamic (or anterolateral) system mediates pain and thermal sensations and some light touch (Fig. 9.1). The receptor input originates from nociceptors, thermoreceptors, and unencapsulated tactile receptors. Thermal sensation is transduced by the temperature-sensitive members of the TRP superfamily (for a review, see Huang *et al.*, 2006); TRPV1–4, TRPA1 and TRPM8 receptors have all been reported to be activated over specific temperature ranges, and are located on the endings of small- to medium-diameter fibers. TRPV1–4 receptors respond to an increase in temperature, whereas TRPA1 and TRPM8 receptors respond to cooling. There are three classes of nociceptors: mechanical nociceptors innervated by Aδ fibers, mechanothermal nociceptors innervated by Aδ and C fibers, and polymodal nociceptors innervated by C fibers. The thermoreceptors and nociceptors are presumed to be free nerve endings.

The afferent nerve fibers of these receptors make synapses on neurons within the dorsal horn of the spinal cord, and the axons of these neurons project to the thalamus via the contralateral spinothalamic tract. Thalamic cells then convey the information to the somatosensory cortex, the limbic cortex, and the insular cortex (Fig. 9.1). The distinction between the two pathways is important clinically as they cross the nervous system at different levels (see Fig. 9.1). Sensation from the face is subserved by comparable trigeminal systems: fibers relaying discriminative touch and kinesthesia synapse in the trigeminal principal and mesencephalic nuclei, which then project to the thalamus via the medial lemniscus. Nociceptive and thermoreceptive afferents from the face connect with the spinal nucleus of the trigeminal, which sends crossed axons directly to the thalamus in the trigeminothalamic tract.

Development of cutaneous receptors and their afferent fibers

Functional receptor–afferent fiber units capable of transducing nociceptive, thermal, and mechanical stimuli into propagated impulses represent an essential first stage for somatic sensation. In this section we will describe the structural, neurochemical, and functional development of the receptors and their afferent projections to the spinal cord.

Structural development

Sensory neurons in the trigeminal and dorsal root ganglia (DRG) are largely derived from a distinct group of cells called the neural crest. The neural crest is a transient structure that arises from the dorsolateral edge of the neural plate just before neural tube closure. The neural tube gives rise to all the cells of the CNS (except for microglia, which are monocytes of bone marrow origin). Neural crest cells migrate widely in the body to appropriate locations to form cranial and spinal ganglia. In the rat, DRG and trigeminal ganglion cells are born over the embryonic (E) period from days 11 to 14 and in humans by about the fourth week of gestational age; these data are not known for sheep. The birth of sensory neurons and growth of their fibers to appropriate peripheral or central targets proceeds in a rostrocaudal direction with events in the lumbar cord lagging behind those in the cervical region; the lag is approximately 20 hours in rodents. Different subpopulations of DRG neurons are specialized for different perceptual modalities. Each functional type has specific molecular characteristics, contains unique sets of ion channels and responds to specific stimuli (for a review, see Marmigère & Ernfors, 2007).

Neurons are produced in excess, and their number is reduced by a process of programmed cell death that coincides with the time at which their axons reach the target tissue. Neurotrophins play a critical role in the survival of different subpopulations of neurons in the dorsal root ganglia, for example: trunk DRG nociceptive neurons require nerve growth factor (NGF) and its receptor TrkA for survival; neurons involved in limb proprioception require NT3 and its receptor Trk C slowly adapting Aβ fibers innervating Merkel's cells; and fibers innervating hair follicles depend on neurotrophin 3 (NT3). Furthermore, approximately 30% of DRG neurons are lost postnatally in mutant mice lacking brain-derived neurotrophic factor

(BDNF); BDNF-dependent neurons probably include large mechanoreceptive neurons (for a review, see Marmigère & Ernfors, 2007). It has been hypothesized that targets for developing neurons produce limiting amounts of these survival molecules so that, on innervation, only those neurons successful in obtaining a certain amount of a particular trophic factor manage to survive. As DRG cells mature they lose their requirement for target-derived trophic support and appear to be sustained by autocrine or paracrine modes of delivery of growth factors, including BDNF (Acheson *et al.*, 1995).

Axons appear to grow directly to their targets in the periphery without sprouting and are possibly directed by chemotropic factors. Innervation of the skin of the hindlimb in sheep occurs by about mid-gestation (Rees *et al.*, 1994a) coinciding with innervation of muscle fibers (Rees *et al.*, 1994b). At this age fibers have penetrated as far as the lower dermis and appear to be using blood vessels for axonal guidance. Large macrophage-like granular cells are associated with developing axons in both sheep (Rees *et al.*, 1994a) and humans (Hogg, 1941). It has been shown that macrophages from embryonic rat brain release NGF in vitro when appropriately stimulated (Mallat *et al.*, 1989). It is possible therefore that these macrophages also play a neurotropic role in skin innervation. Fibers can be seen penetrating the epidermis at 101 days (about 0.68 gestation) with more extensive branching at 110 days. In the rat, facial innervation begins on day E13 and hindlimb innervation takes place over E15–E19. In humans, cutaneous innervation of the face, shoulder, axilla, and thigh has begun at eight weeks of gestation. In both rats and humans, cutaneous nerve terminals initially form a dense plexus penetrating into the fetal epidermis. With time, the fibers withdraw and reduce in density as receptors such as Meissner's corpuscles appear and become innervated some time after the initial innervation of the skin. Hair follicles are innervated later in gestation – at about 100–106 days in the sheep, postnatal day (P)7 in the rat, and 22–24 weeks of gestation in the human.

In fetal sheep, dorsal root afferent fibers have begun to penetrate the gray matter of the dorsal horn by 56 days of gestation (Fig. 9.2*a*) and by 67 days, innervation of the motor neuron pool is established (Fig. 9.2*b*); there is a marked increase in the number of fibers and the extent of their arborization in the dorsal and ventral horns with increasing gestational age (Fig. 9.2*c*, *d*). From ultrastructural studies we have shown that afferent fibers (immunoreactive for substance P and calcitonin gene-related protein [CGRP], see below) form synaptic connections with dorsal horn cells within a few days of their arrival in layer 1 of the dorsal horn (Rees *et al.*, 1994b). In the rat, dorsal root fibers first reach the lumbar cord at E12, travel rostrocaudally in the "bundle of His" and begin to send collaterals into the dorsal gray matter at E15 (Fitzgerald *et al.*, 1991; Mirnics & Koerber, 1995). The first sensory fibers to grow into the cord are the large-diameter myelinated afferents, some of which are 1A muscle afferents projecting towards the motor neuron pool in the ventral horn and some are Aβ cutaneous afferents that remain in the dorsal horn (Fitzgerald *et al.*, 1991). Some days later (E19–E20), smaller-diameter unmyelinated C fibers grow into the substantia gelatinosa (layer II). The organized somatotopic projections and laminar location of C fibers is established early in development and requires little refinement to match that in the adult (Mirnics & Koerber, 1995). Although somatotopy (i.e., the mapping of the body's surface sensations onto a structure in the brain) is established early in gestation for A fibers, laminar location is not (Fitzgerald *et al.*, 1994). A-type fibers are initially found throughout layers I–V, including the substantia gelatinosa (layer II), which later becomes the major projection domain of C fibers. By postnatal day 22, A fibers have withdrawn to layers III–V. It is not yet certain what causes this withdrawal but it is possibly due to competitive interactions between the A fibers and the later arriving C fibers (Coggeshall *et al.*, 1996), perhaps triggered by neurotrophic factors or a mismatch between activity levels in A fibers and neurons in the substantia gelatinosa. In another study of prenatal development of the central projections of primary afferents in the rat, Mirnics and Koerber (1995) concluded that the initial penetration of the gray matter is a target-independent process; peripheral innervation is not invariably the stimulus for fiber ingrowth into the cord, and the establishment of topography and modality in the cord is likely to be target-dependent and with a postnatal component responsible for the subtle refinements of these projections, probably requiring activity-dependent mechanisms. Our findings in the sheep concur with their observations in that central projections of afferent fibers grow into the dorsal horn prior to innervation of the distal hindlimbs.

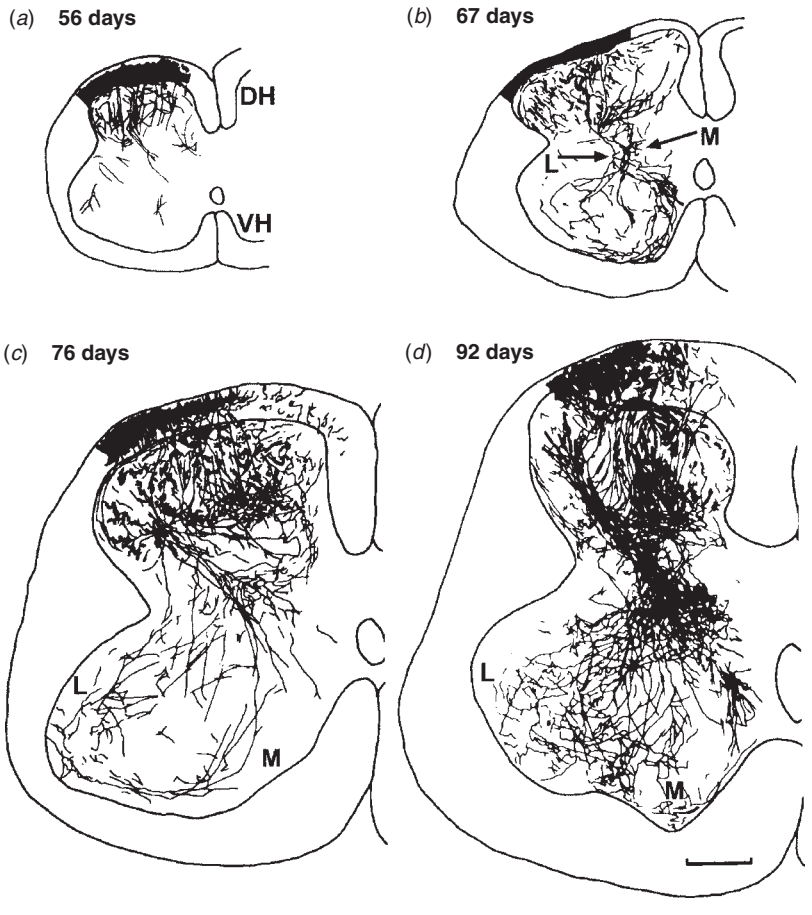

Fig. 9.2 Drawings of the distribution of primary afferent fibers stained with the neural tracer biocytin in the developing fetal sheep spinal cord. At each gestational age, the section of cord that contained the greatest number of stained fibers has been selected for presentation. (*a*) At 56 days fibers have entered the spinal cord and penetrated as far as the intermediate zone with a few fibers projecting into the ventral horn. (*b*) By 67 days primary afferents converge in the intermediate zone (arrows) before they spread out and extend into the medial (M) and lateral (L) motor neuron pools. With advancing gestational age at (*c*) 76 days and (*d*) 92 days, the number of primary afferents entering the spinal cord and following the trajectory described above has increased. The density of innervation of both the medial and ventral motor neuron pools increases concomitantly. Scale bar = 300 μm. DH, dorsal horn; VH, ventral horn. (From Rees. S., Rawson, J., Nitsos, I., *et al.* (1994b), *Brain Research*, **642**, 185–98, with permission).

Neurochemical development

In addition to the development of the anatomical substrate for sensory perception, the development of neurochemical messengers in these pathways is also a prerequisite for a functional system. Neuropeptides, excitatory amino acids, and monoamines have all been implicated in sensory transmission mechanisms in the spinal cord as neurotransmitters or neuromodulators. Substance P is found in small-diameter DRG cells and Aδ and C fiber sensory afferents, and CGRP is found in small to large DRG cells and Aα, Aβ, Aδ, and C fibers. The excitatory amino acid glutamate (GLU) has been implicated as a neurotransmitter for at least some of the primary afferent fibers (Jessell *et al.*, 1986). In the sheep, it was seen that afferent fibers immunoreactive for substance P or CGRP were present in Lissaeur's tract and layer I of the dorsal horn by 56–61 days of gestation (Fig. 9.3), that is within a few days after their arrival in the dorsal horn. The first appearance of peptides in the peripheral endings of primary afferent fibers in the skin of the fetal sheep is several weeks later at 85 days, at least 10 days after there is evidence of their presence using conventional histological techniques. Similarly, in the rat, is was reported that peptides were not present in the skin until some days after axons had innervated the dermis (Marti *et al.*, 1987). In the human, a few CGRP-IR nerve fibers have been seen in the skin a few days after the time of skin innervation at seven weeks but appear more consistently at 17 weeks of gestation (Terenghi *et al.*, 1993). CGRP is first detected in the dorsal horn at E17 in the rat and at week 10 in humans (Marti *et al.*, 1987). Fibers immunoreactive for substance P first appear in the dorsal horn at E16–E18 in the rat and at 11 weeks of gestation in the human. In terms of the total length of gestation, the first appearance of these peptides is at 28% of gestation in humans, 38% in sheep, and 79% in rat. In humans and sheep therefore, peptides are present for a considerable time during the

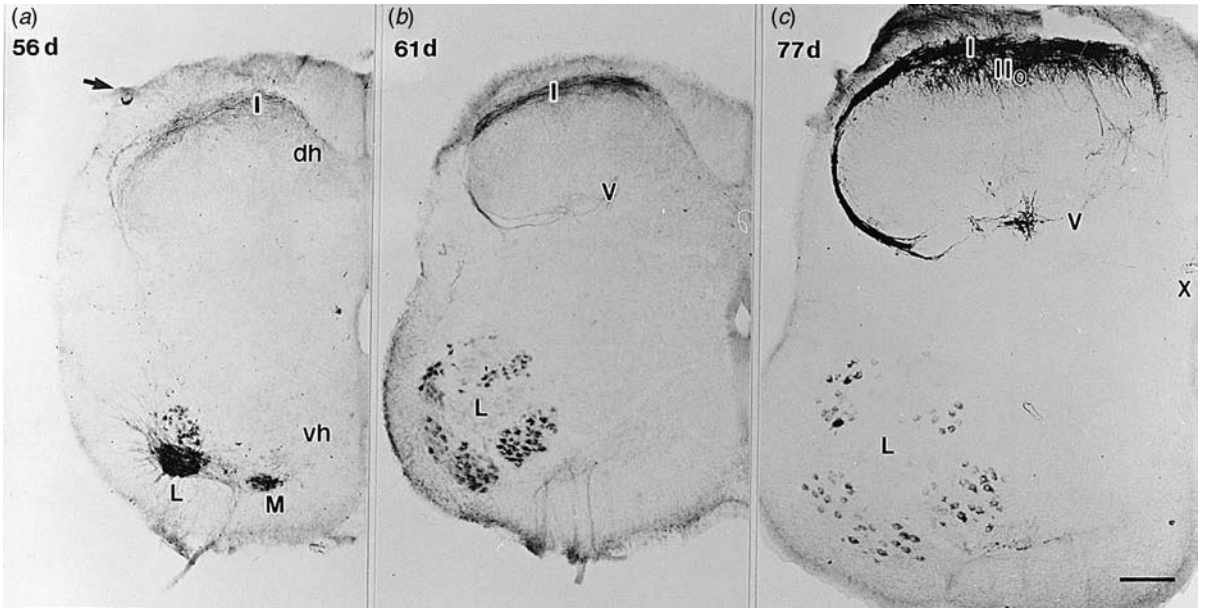

Fig. 9.3 Photomicrographs of immunoreactivity (IR) for calcitonin gene-related protein (CGRP) in the spinal cord of fetal and postnatal sheep. (*a*) At 56 days, CGRP-IR was seen in layer I, the dorsolateral funiculus and in the tract of Lissauer (arrow). Medial (M) and lateral (L) motor neuron pools in the ventral horn were strongly immunoreactive. (*b*) By 61 days, fibers immunoreactive for CGRP were now present in the central region of layer V. At this stage, intensely staining motor neurons were now concentrated in the lateral motor neuron pool. (*c*) At 77 days, CGRP-IR fibers were found to extend into the central region of layer II but this was predominantly in the outer layer of this layer (II_0). A few fibers were also present in layer X, the region surrounding the central canal. Scale bar = 170 μm. dh, dorsal horn; vh, ventral horn.

intrauterine development of these pathways. It is possible that they play an important role in the normal growth and differentiation of the nervous system in addition to their role as neurotransmitters or neuromodulators. The reason for the delayed appearance of peptides in the periphery is not certain but could be partly due to slower axonal transport in the peripheral branch of the developing sensory fiber.

Functional development

In fetal sheep, the earliest age at which natural cutaneous stimulation could evoke activity in the DRG and dorsal horn was shown to be at 75 days gestation, i.e., at about mid-gestation (Rees *et al.*, 1994a). These responses were low threshold, with light stroking or indentation of the skin being an adequate stimulus to evoke a brief discharge in DRG cells and in dorsal horn cells. Receptive fields were initially large, reducing in size with advancing gestational age. However, the overall impression we obtained from this study was that there was little change in the response patterns of the receptors with increasing gestational age. Although the action potentials of DRG cells tended to increase in size and decrease in duration and could be evoked at a higher frequency, there were no signs of an obvious shift in basic response types.

Thus, it appears that even very immature receptors are capable of responding to specific stimuli and further receptor development serves to enhance the fidelity of stimulus-response coding as indicated in Fig. 9.4*a*. This impression was confirmed in the case of the wool/hair follicle receptors where it was possible to correlate functional and structural development. These afferents could be selectively stimulated by de ecting the wool/hair shaft and receptor units could be readily identified in microscopic analysis of the skin. The earliest response elicited from these afferents was phasic on/off and occurred at a stage when the follicle innervation was simple and just established. This pattern persisted throughout gestation even though there were major changes in the complexity and conformation of follicle innervation. It is possible that the basic response properties of a receptor are already established at this stage of axon terminal formation and that further structural development refines the transduction process by shaping the sensitivity and firing frequency of the receptor-afferent unit.

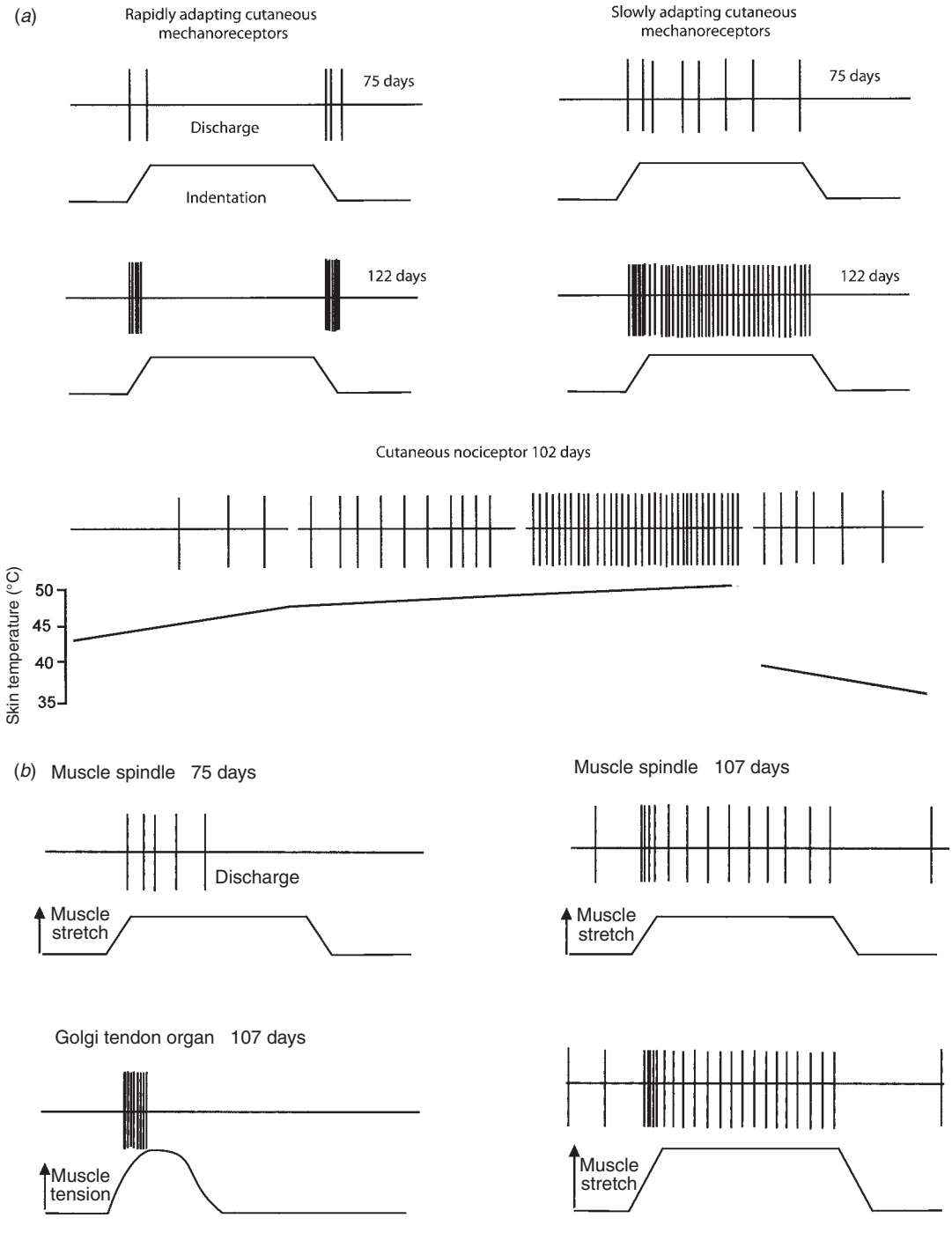

Fig. 9.4 (*a*) Diagrammatic illustrations of responses of developing cutaneous receptors in the sheep fetus. The top two traces show typical responses of receptor afferents to skin indentation at 75 days gestation (term ~ 147 days). The afferents signal the stimuli but do not respond with high-frequency discharge. The traces underneath show responses of comparable receptor types later in gestation where there is an improved ability to code both changing and steady pressure on the skin. The lowermost traces indicate the ability of a fetal cutaneous nociceptor to respond to noxious heat. This afferent was silent at normal skin temperature but started to discharge when the skin was heated to about 45 °C and fell silent again when heating was removed. (*b*) Examples of fetal muscle receptor responses. The upper left traces indicate the response of an immature muscle spindle afferent to a muscle stretch. The unit was responsive but only during the initial phase of the stretch and failed to signal the duration of the applied stretch. The sets of traces on the right show responses of a more mature muscle spindle at 107 days gestation. Here the afferent could signal a steady length change in the muscle and could also code muscle length by its discharge rate as can be seen from the lower panel response where a greater stretch was applied to the muscle. Golgi tendon organs also develop functionally in utero as shown in the traces on the lower left, which indicate an afferent responding to the rise of tension generated by a muscle twitch.

Throughout gestation in the sheep, the majority of cells in the lumbar dorsal horn and DRGs responded to a low-threshold cutaneous stimulus, with cells responding to a high-threshold mechanical stimulus being encountered less frequently. In the rat, primary afferent fibers develop receptive fields to natural stimulation at E17 (Fitzgerald, 1987b) with dorsal horn cells developing receptive fields two days later at E19 (Fitzgerald, 1991), the delay possibly representing the time needed for maturation of central synaptic connections. These results are consistent with behavioral studies in which stimulation of the plantar surface of the hindlimb produces a withdrawal re ex at E19 (Narayanan *et al*., 1971). In comparison with sheep, the earliest responses in the rat from the skin of the hind paw were high threshold, requiring firm pressure to produce a few spikes (Fitzgerald, 1987b). The authors suggest that this might re ect an immaturity of synaptic connections rather than any preferential input from afferent nociceptors. In fact, it has been shown in the rat that all direct cutaneous-evoked dorsal horn activity in the first postnatal week (and presumably before birth) results from activation of A fibers (Jennings & Fitzgerald, 1998). For example, low-threshold mechanical stimulation can evoke *c*-fos expression in the first postnatal week (Jennings & Fitzgerald, 1996). The formation of C-fiber synaptic connections is almost entirely a postnatal event in the rat (Fitzgerald, 1987a), with C fibers not becoming active until about P10.

As mentioned above, in the neonate, A fibers terminate in the dorsal horn in layers I–V withdrawing to layers III–V after about postnatal week 3; in the adult layer II is the domain of C fibers. A fiber input in the neonate produces postsynaptic excitatory effects in dorsal horn cells, particularly sensitization, not seen in the adult; this might be important for normal sensory function in the developing mammalian system (Jennings & Fitzgerald, 1998). Large-diameter primary afferent fibers in neonatal rats therefore make synaptic contacts with presynaptic targets that presumably process nociceptive information (Coggeshall *et al*., 1996). Coggeshall and colleagues (1996) suggest that in ameliorating pain in neonates it might be more important to block low-threshold sensory input whereas in adults it would be more important to block high-threshold inputs. There is no direct evidence yet that a similar organizational change of A fiber input occurs in humans. If it does occur, we consider that it would most likely take place at some stage during fetal life in long gestational species such as sheep and humans where the spinal cord is far more developed at birth than it is in the rat. Clear lamination in the cord is not seen until E17 in the rat while it is evident at week 13 in the human fetus and at about day 61 in the sheep fetus. It is known that cutaneous re exes in the newborn human, as well as in the rat and kitten, are exaggerated compared with the adult, as exor re exes can be elicited by light touch rather than by the noxious stimuli required in the adult (Ekholm, 1967; Andrews & Fitzgerald, 1999). This could, of course, be due to other aspects of developing spinal cord circuitry such as lack of descending inhibitory control rather than, necessarily, reorganization of input to layer II.

There are further differences between species in the functional development of cutaneous sensory input to the spinal cord. In the rat, spontaneous activity of cutaneous afferents in the DRG is present from E16, peaking at E18–19 (Fitzgerald, 1987b) and at E20–21 some dorsal horn cells displayed responses that outlasted the initial stimulus by 10–15 seconds (Fitzgerald, 1991).These observations were not made in the sheep fetus possibly due to the different experimental conditions, species differences in the development of electrical properties of sensory neurons, or in the maturation of synaptic connections within the spinal cord.

In sheep we observed that the earliest responses of dorsal horn cells were sluggish and the action potential slow. It has been suggested that during fetal development there are considerable changes in the ionic dependence of the inward current of the action potential in sensory neurons (Spitzer, 1994). This change frequently involves the gradual conversion of Ca^{2+}-dependent impulses of long duration into fast Na^{+}-mediated potentials. This conversion could explain the change in the shape of the action potential observed in sheep. It is not certain what role this alteration in ionic dependence might play in neural development, but it has been suggested that calcium transients might be important components of the pathway resulting in gene expression in differentiating cells (Spitzer, 1994).

In other studies exogenous application of the inhibitory neurotransmitter -aminobutyric acid (GABA) results in depolarization of neurons in the superficial dorsal horn in the first postnatal days in rats, and that by the end of the first postnatal week GABA induces a hyperpolarizing current, similar to that seen in adult neurons (Baccei & Fitzgerald, 2004).

This type of response has been reported in neonatal neurons in other brain areas and is thought to reflect change in the direction of flow of Cl^- as the membrane potential develops because of increasing activity of Na^+/K^+-ATPase pumps. In immature neurons with low membrane pump activity, Cl^- tends to flow outward through the GABA receptor channel (hence, depolarization), but with greater separation of Na^+ and K^+ due to increased activity of the membrane pump, Cl^- tends to move into the neuron (hence, hyperpolarization).

The development of thermoreceptors and thermal sensitivity appears to be of relatively late onset. Although both cold and TRPV1 agonists can evoke Ca^{2+} currents in DRG neurons from E12.5 in the mouse, the specific TRPM8 (cold) receptor agonist menthol is not effective until just before birth (Hjerling-Leffler *et al.*, 2007). The authors suggest that sensory neurons expressing TRPM8 derive from TRPV1-expressing sensory neurons – in essence, that a switch in phenotype occurs. At central primary afferent terminals in the postnatal rat, a similar developmental progression is seen with capsaicin, a TRPV1 agonist, which is able to evoke an increase in glutamate release from birth but menthol, the TPRM8 agonist, is only able to induce a similar response from P10 (Baccei *et al.*, 2003).

Development of muscle afferents and their fibers

Structural development

The sequence of events involved in the innervation and structural development of mammalian muscle spindles has been well established in several species (Barker & Milburn, 1984) but much less is known about the functional development of muscle spindles in utero. Our work in the sheep (Rees *et al.*, 1994b) has shown that the timing of the structural and functional development of muscle receptors and their afferent connections is closely associated with both the innervation of extrafusal muscle fibers and the appearance of early fetal movements. Immature afferent innervation of muscle spindles was evident at 67 days of gestation in the hindlimb of fetal sheep (i.e., at approximately 46% gestation). Innervation also occurs at about this time. At 67 days muscle spindle innervation was in the form of a fine network of fibers extending from two major branches; well-formed annulospiral windings were not present and did not appear until approximately 83 days of gestation. With further development there was a progressive increase in the length of the spindle and the complexity of its innervation, until at approximately 127 days it had largely acquired the adult form. Fusimotor innervation of intrafusal fibers was evident from 83 days of gestation although specialized endings did not form until about 100 days. Muscle spindles in more rostral muscle groups such as the intercostal muscles develop earlier (~47 days) than in the hindlimbs.

Functional development

As with cutaneous receptors, the earliest age at which activity could be evoked in the DRG to muscle stretch was at approximately 75 days of gestation (Rees *et al.*, 1994b). It has been reported briefly that muscle afferents in the fetal rat discharge in response to a small change in limb position from E17 onwards but there has not been an extensive developmental study in the fetal rat.

As mentioned above, muscle spindles in the sheep at 75 days are small and their afferent fibers are just beginning to form the annulospiral winding characteristic of the mature spindle. Since it was possible to evoke activity in spindle afferents with an applied stretch at this age (Fig. 9.4*b*) it appears that the annulospiral formation is not a necessary requirement for the generation of a response. At this age, however, the spindle does not appear to be capable of generating a consistent tonic response at resting muscle length, nor does it respond to stretch with a sustained train of discharges (Fig. 9.4*b*). Within two weeks of the onset of activity (that is by about 87 days) responses to stretch are easier to elicit. Receptors begin to develop a tonic discharge at resting muscle length, and stretch-evoked responses displayed clear static and dynamic sensitivities with the static component predominating. As indicated above, spindles are still relatively immature but annulospiral windings are beginning to form and, in a few spindles, are well developed. In general, these results are supported by findings in the kitten, in which afferent responses demonstrating several features of the adult response are present well before an annulospiral structure has developed.

Spontaneous and evoked activity became more stable with increasing gestational age (up to term). The frequency of the tonic discharge increased from 10 Hz at 87 days to 35 Hz at 127 days, and both static and dynamic sensitivities become more pronounced.

Parallel to this functional development was the progressive increase in the complexity of the spindle innervation. There were, however, no specific structural changes which could be correlated with particular aspects of functional development. Responses typical of Golgi tendon organs, where the afferent responds to the tension generated by a muscle twitch, were also evident in utero (Fig. 9.4*b*).

Fusimotor innervation of intrafusal fibers was evident from 83 days of gestation, although specialized endings did not form until about 100 days. Conduction velocity of all muscle receptor afferents then increased markedly during the last four weeks of gestation. This is not unexpected, since a precocious animal such as the sheep needs to have well-developed motor control immediately after birth.

Experiments with the neural tracer biocytin in fetal sheep revealed afferent projections to motor neuron pools by 67 days of gestation (Fig. 9.2*b*), that is just before muscle receptors had started to respond to stretch. The possibility therefore exists for even the earliest muscle receptor activity to in uence motor neurons, and presumably other target neurons in the spinal cord. Furthermore, there was evidence of innervation of extrafusal muscle fibers by 67 days of gestation. Thus the neural pathways required for re ex activity involving muscle spindles are present from early in gestation.

Spontaneous activity is a feature of the developing CNS, and it may have an important role in establishing and refining specific connections between nerve cells. In the spinal cord spontaneous activity is present in the embryo and fetus, and in fetal sheep movements can be observed with ultrasound from about 60 days.

Development of ascending pathways from the spinal cord to the brain

The most detailed information on the development of somatosensory projections to the thalamus and cerebral cortex has been obtained from the rat, and it appears that the first ascending projections reach their targets in synchrony and then undergo a phase of maturation in which the terminal arbors are fully developed. The cells of the ventral thalamus are born on E13 and have migrated and settled in position by E16–17 (Altman & Bayer, 1979). The first afferents from the trigeminal system (Leamey & Ho, 1998) and dorsal columns arrive in the thalamus at E17. After their arrival, the fibers continue to develop terminal arbors and at birth have formed a profuse innervation of the thalamus that continues to develop over several weeks postnatally. Interestingly, it appears that extensive maturation of these fibers is not required before they can transmit information, as they quickly establish functional synapses and are capable of activating thalamic cells at E17 (Leamey & Ho, 1998).

Fibers start to leave the thalamus at E16, to arrive below the developing somatosensory cortex at E17. The growth of thalamic axons into the cortex and their further maturation coincides with the establishment of structural and neurochemical patterns that are characteristic of cortical somatosensory representation of the body parts, which are clearly evident by the day of birth (Schlaggar & O'Leary, 1991). The factors that guide and control the formation of these thalamocortical connections are still being elucidated. It has been suggested that the cortical subplate may play an important role in the initial guidance of thalamocortical axons into the cortex; the subplate consists of a stratum of transient cells located below the developing cortical plate. The thalamocortical fibers in some sensory systems such as the visual system in the cat (Ghosh & Shatz, 1992) remain at the level of the subplate for a time, as if waiting for a critical recognition process to occur before growth into the cortex itself can proceed. Some studies propose that this process requires impulse activity (Catalano & Shatz, 1998), although there is not full agreement on this notion (Molnar *et al.*, 2002).

Somatosensory thalamocortical fibers, at least in some species such as the wallaby (Pearce & Marotte, 2003), do not appear to wait at the subplate but grow directly through it to the developing cortex. It has been suggested that the maturity of the cortex itself may be a factor in regulating the ingrowth of thalamocortical projections (Catalano *et al.*, 1991). Kanold and Shatz (2006) recently suggested that subplate neurons also regulate the molecular machinery required to establish the crucial balance between excitation and inhibition in layer IV (which receives the thalamic afferents), thereby in uencing the outcome for ocular dominance plasticity in the visual cortex. For further discussion of the possible roles of the subplate in axon guidance see Allendoerfer and Shatz (1994), Molnar and Blakemore (1995), and Molnar *et al.* (2002).

The overall picture which emerges from studies on the rat is that the substrates underlying discriminative sensation from the face and body start to develop just

about halfway through gestation and progressively mature over several weeks of postnatal life with the refinement of afferent connections with the thalamus and cortex. In animals with a long gestation period these stages of development are almost certainly attained in utero. For example, in humans, cortical layer IV begins to differentiate at about 18–20 weeks of gestation. Synapses begin to appear in the cortex shortly afterwards. Cortical evoked responses to somatosensory stimulation can be recorded at 25 weeks of gestation but are diffuse and largely undefined. By 29 weeks a more mature response with a primary negative component has developed (Klimach & Cooke, 1988). In the sheep fetus, stimulation of the limbs evokes responses in somatosensory cortex at 120 days of gestation (Cook *et al*., 1987) indicating that the pathway to the cortex has already formed by this stage and is capable of transmitting somatosensory input. Very little is known about the development of the spinothalamic pathway which transmits information from nociceptors and thermal receptors. In the fetal sheep, fiber terminals from lumbar spinal cord cells appear in the thalamus between 120 and 130 days of gestation (Rawson & Rees, 1994, unpublished observations) indicating that spinothalamic projections are well established before birth in this species.

Functional imaging techniques have recently emerged as a highly advantageous means of studying human cortical development postnatally. In relation to lateralization of somatosensory input to the cortex, Erberich and colleagues (2006) have demonstrated that at 38–49 postnatal weeks there is considerable bilateral activation of the cortex and thalamus in response to passive stimulation of cutaneous and proprioceptive receptors in the hand; by three to nine months all responses are contralateral to stimulation. Therefore it appears that the refinement of somatosensory systems and lateralization must occur in the postnatal period.

Development of descending control systems

Transmission of all modalities of somatosensory input is potentially subject to control by descending systems that "gate" or modify the in ow of information to the thalamus or cerebral cortex according to functional requirements. The primary somatosensory cortex projects densely to the ventroposterior thalamic complex where the medial lemniscus terminates and this projection presumably has an important role in the final regulation of transmission of tactile and proprioceptive inputs to the cerebral cortex. In addition, the cortex also provides direct projections to the dorsal column nuclei, to the dorsal horn of the spinal cord and there is also a projection to Clarke's column, which has been shown physiologically to control proprioceptive input from the hindlimb (McIntyre *et al*., 1989). Cortical input to the thalamus develops in utero, and a few projections from layer VI of somatosensory cortex to the thalamus are present in the rat as early as E16 (Catalano *et al*., 1991). However, it remains unclear when the cortex starts to in uence transmission from the thalamus.

Spinal transection in rats has little in uence on the re ex activity of the spinal cord before P12 (Weber & Stelzner, 1977) and in kittens before about two to three weeks postnatal age (Ekholm, 1967). In humans, it seems that descending control from the cortex is also likely to develop after birth and mature over a prolonged period given that the corticospinal tract is poorly myelinated at birth and takes several years to develop fully. Indeed, one of the first obvious signs of cortical modulation of sensory transmission in the spinal cord occurs at about eight to nine months of age when the plantar re ex changes from extensor to exor.

The transmission of information from nociceptors can be potently inhibited by a descending antinociceptive pathway that projects to the spinal nucleus of the trigeminal and the dorsal horn of the spinal cord; this is discussed fully elsewhere in this volume. A major component of this pathway originates in the raphe nuclei of the brainstem, which provide serotonergic fibers that descend through the dorsolateral funiculus of the spinal cord, and acts via release of endogenous opioid peptides. Endogenous opioids and descending projections from the brainstem start to develop at an early intrauterine stage in animals with a long gestation period. Enkephalin-containing cells and fibers and opioid receptors are present in the human spinal cord at 14 weeks of gestation (Charnay *et al*., 1984) and in the sheep fetus projections from the raphe nuclei are present in the lumbar spinal cord at 60 days' gestation, which is less than halfway to term (Rawson & Rees, 1994, unpublished observations).

The most detailed information about development of this pathway has been obtained from the rat. Serotonergic fibers first appear in the spinal cord just before birth at E18 and continue to mature and

establish connections in the dorsal horn, attaining an adult-like pattern at P21 (Bregman, 1987). Opioid peptides and receptors also start to appear just before birth but the opioid system undergoes a prolonged phase of development and maturation that extends well into postnatal life and involves changes in the expression of different peptides and receptors as well as age-dependent functional changes in the receptor response to opioids (Rahman *et al.*, 1998; Nandi & Fitzgerald, 2005). This largely postnatal development of important components of the antinociceptive system in the rat correlates well with findings that stimulation of the dorsolateral funiculus of the spinal cord (a serotonergic pathway) only starts to evoke inhibition in dorsal horn neurons after birth at P10 and inhibition becomes comparable with that seen in the adult by P22 (Fitzgerald & Koltzenburg, 1986; Giordano, 1997). The sensitivity of C-fiber-evoked responses to inhibition by morphine in the rat also develops postnatally and peaks at about P21.

Thus in the rat, descending fibers and opioids are potentially capable of modulating nociceptive transmission by the first few weeks of postnatal life, but it is not yet clear for the rat or other species when the antinociceptive system begins to function under normal control. In other species in which gestation is longer, the placenta produces large amounts of progesterone that, on entering fetal blood, can be modified to produce pregnane derivatives that act at $GABA_A$ receptors in the fetal brain and suppress brain activity. $GABA_A$ receptors are strongly expressed throughout the fetal sheep brain with expression rising with advancing gestation, and, although receptor concentrations differ between brain regions, adult concentrations are attained by normal term in most regions (Crossley *et al.*, 2000). In sheep, the placental supply of steroid precursors such as pregnenolone and progesterone contributes to the synthesis of $GABA_A$ receptor agonist compounds such as allopregnanolone in the fetal brain (Crossley *et al.*, 1997). The high concentrations of these steroids, relative to the newborn, found in the fetal brain in late gestation provides a mild physiological suppression of fetal CNS activity (Crossley *et al.*, 1997) and reduces the sensory responsiveness of the fetus to external stimuli (Nicol *et al.*, 1998). Thus, the positive modulation of GABAergic transmission in the fetal CNS by neurosteroid agonists may contribute to the obtunding of sensation and to antinociception during fetal life.

Emergence of functional systems in utero

The onset of cutaneously evoked activity from the hindlimb at E19 in the rat and 75 days of gestation in the sheep occurs at approximately similar stages of spinal cord development. In terms of the gestational period, however, sensory pathways first become functional at 50% of gestation in the sheep but not until 87% of gestation in the rat. Muscle spindles in the hindlimb also respond to stretch at about 75 days of gestation in the sheep. As there is a rostrocaudal sequence of development, input from the face and snout in the sheep reach the spinal cord (and cortex) even earlier in gestation. As the sheep is a prenatal developer, required to exist independently at birth, it is not unexpected that these pathways begin to function earlier in the sheep than they do in an altricial species such as the rat.

The emergence of important features of somatosensory function in humans is summarized in Table 9.1 and has recently been reviewed by de Vries and Fong (2006). Observations of the human fetus with real-time ultrasound have shown that the first discernible movements are detectable at seven to eight weeks of postmenstrual age (de Vries *et al.*, 1982). Movement of individual limbs occurs at about nine to ten weeks with more complex movements such as sucking and swallowing evident at 12–14 weeks. More recent ultrasound studies have suggested that handedness, the most prominent manifestation of behavioral lateralization in humans, develops from about ten weeks of gestational age (Hepper *et al.*, 1998). Fetuses showed a highly significant preference for arm movement with 85% exhibiting more right than left arm movement. In aborted fetuses it has been possible to evoke movements from light stimulation in the perioral region from six to seven weeks (gestational age) (Hogg, 1941). This indicates that low-threshold mechanoreceptors are operative at this age and have established functional connections with motor neurons. Structural connectivity has been confirmed in ultrastructural studies where synapses between sensory neurons, interneurons, and motor neurons have been demonstrated at five and a half to six weeks of gestation (Okado, 1981). By 14 weeks, stimulation of most areas of the body surface can evoke re ex responses.

Why would it be necessary for these pathways to become functional so early in gestation? It is widely accepted that thalamocortical pathways initially

Table 9.1 Emergence of fetal behavior in humans

Behavior	Methods of assessment	Age of first appearance	Reference
Movement in response to peripheral stimulation	Cutaneous stimulation, ex utero fetus	6–7 weeks	Hogg (1941)
First discernible movement	Ultrasound	7–8 weeks	de Vries *et al.* (1982)
Isolated arm movement	Ultrasound	9–10 weeks	de Vries *et al.* (1982)
Jaw opening	Ultrasound	10–11 weeks	de Vries *et al.* (1982)
Stretch	Ultrasound	10–11 weeks	de Vries *et al.* (1982)
Evidence of handedness, right predominance	Ultrasound	10 weeks	Hepper *et al.* (1998)
Marked increase in changes in fetal position	Ultrasound	10 weeks onwards, peaked 15 weeks	de Vries *et al.* (1982)
Hand sensitive to touch	Cutaneous stimulation, ex utero fetus	10.5 weeks	Humphrey (1978)
Sucking and swallowing movements	Ultrasound	12–14 weeks	de Vries *et al.* (1982)
Re ex activity evoked by stimulation of most body areas	Cutaneous stimulation, ex utero fetus	14 weeks	Humphrey (1978)
Cutaneous withdrawal re ex	Cutaneous stimulation, premature infant	26 weeks	Andrews & Fitzgerald (1994)
Cortical evoked responses to somatosensory stimulation	Cutaneous stimulation, premature infant	25–29 weeks	Klimach & Cooke (1988)
Coordinated facial actions indicative of pain	Heel prick, premature infant	26–31 weeks	Craig *et al.* (1993)
Peak incidence of exion and stretch during gestation	Ultrasound	28–31 weeks	Kozuma *et al.* (1997)
Lateralized head position (mainly to right)	Ultrasound	38 weeks	Ververs *et al.* (1994)

develop by activity-independent mechanisms involving guidance molecules such as Eph receptors and ephrin ligands and members of the Wnt signaling pathway (reviewed by Price *et al.*, 2006). Across the cortex graded concentrations of signaling molecules and transcription factors (for example, Pax6 and Emx2) appear to be critical in establishing cortical maps of the topography of sensory surfaces. Later in development, stimulus-driven, activity-dependent mechanisms are thought to be the driving force in refining cortical connections although the exact role of neural activity is unclear. Early studies on the visual system have demonstrated that cortical ocular dominance columns formed under the in uence of retinal activity (LeVay *et al.*, 1978). However, recent studies in the ferret, using new tracing methods (Crowley & Katz, 2000), have challenged the notion that activity is required, at least in this species.

In the somatosensory system studies on knockout mice with cortical barrel field deficits support a role for activity (Abdel-Majid *et al.*, 1998; Inan *et al.*, 2006). On the other hand, thalamocortical connections in mice with a disruption in the gene encoding SNAP-25, a molecule involved in synaptic transmission, appear to develop normally in the absence of evoked activity, at least up to birth (Molnar *et al.*, 2002). In the wallaby, a marsupial mammal born at an extremely early stage of development, activity-dependent mechanisms do not appear to operate until relatively late in the development of thalamic connections with the primary somatosensory cortex and it is not certain how important they are for normal development (Leamey *et al.*, 2007).

Currently the only evidence of rearrangement of synaptic connections during development of the primary afferent innervation of the spinal cord is the withdrawal of A fibers from layer II during the first three weeks postnatally in the rat. In any event, neural activity could play an important role in the reinforcement of pathways once they have formed. Certainly in the adult mammalian somatosensory system, activity in primary afferents plays an important role in shaping and maintaining receptive fields. This is well illustrated by findings that after loss of input from a part of the body, the cortical receptive fields or representations of adjacent parts expand and occupy the area where the denervated part was represented. Taken together, there

is no question that cortical maps can be altered by neural activity, but it remains to be seen how essential activity is, in general, for the construction of early maps during brain development.

One role of early afferent activity may be in the development of circuitry such as the exor re ex response. In rats, the development of exor re exes involves a learned component referred to as "somatosensory imprinting," the tuning of which depends on spontaneous movements during sleep (Schouenborg, 2008). Furthermore, the first postnatal week is a critical period for somatosensory imprinting, in that blockade of both high- and low-threshold afferents results in many more erroneous connections in sensorimotor circuits.

It must be pointed out that input from somatosensory receptors is utilized by functional systems other than those subserving conscious perception. The cerebellum receives massive somatosensory input from several pathways originating in the spinal cord and brainstem. There are somatosensory inputs to the hypothalamus, vestibular nuclei, inferior colliculus, parabrachial nuclei, and respiratory control centers of the brainstem. In the same way that the development of the re ex circuitry of the spinal cord depends on input from peripheral receptors, other circuits in the CNS might require somatosensory input during their development.

Within the uterus, stimulation of low-threshold cutaneous mechanoreceptors in the fetus could occur with movement of the amniotic uid, contractions of the uterus, or from self-stimulation as limbs touch the body and the fingers touch the face. This could then result in re ex contraction of muscles and activation of Golgi tendon organs and muscle spindles. Muscle spindles would also be activated by changes in limb position and these receptors also generate resting or background activity as they develop. Ultrasound studies have demonstrated that there is a marked increase in the rate of change in position of the fetus from about 10 weeks onwards, peaking at 15 weeks, after which time there is a reduction, perhaps due to spatial restrictions on fetal movement (de Vries *et al.*, 1982), the development of descending inhibition, or increased suppression of CNS activity by neuromodulators such as neurosteroids, as mentioned above.

Whether a fetus can "feel" pain in utero is controversial. Some definitions of pain imply that the brain must achieve a certain level of neural functioning as well as having prior experience before pain can be understood (see Lloyd-Thomas & Fitzgerald, 1996). This question is dealt with fully elsewhere in this volume, but such a definition would clearly exclude the fetus from "feeling" pain. It is clear, however, that the fetal nervous system can respond to potentially damaging stimuli by the beginning of the last trimester, and from about 26 weeks of gestation prematurely born infants show a measurable withdrawal (exion) re ex to noxious stimulation suggesting that the nociceptive afferent input to the spinal cord is present and can function at this age (Andrews & Fitzgerald, 1994). It is reasonable to assume that such responses could also occur in utero, although it is di cult to envisage that nociceptor activation occurs during fetal life except perhaps during fetal surgery or during maneuvers such as amniocentesis when the fetus may be pricked by a needle. If the transient connections between A fibers (which originate from low-threshold mechanoreceptors) and layer II neurons described above for the rat also exist in humans they might provide a means of generating input to nociceptive pathways during critical phases of development when none is likely to come from nociceptors themselves. In addition to spinal re exes, infants delivered at 26–31 weeks show coordinated facial actions indicative of pain such as brow bulging, eyes squeezed shut, and open mouth in response to a heel prick (Craig *et al.*, 1993).

Recent studies using near infrared spectroscopy in premature human infants provide evidence for activation of somatosensory cortex in response to heel lance (Slater *et al.*, 2006) or venipuncture (Bartocci *et al.*, 2006) from infants from as young as 25 weeks' gestation. Nevertheless, it is still not certain whether such nociceptor activation in the premature infant or fetus is perceived as pain, given that sensory impulses do not evoke a mature cortical response until ~29 weeks of gestation (Klimach & Cooke, 1988). Any assessment of pain in the neonate must also take into account that the infant nervous system has a very low threshold for excitation. In the case of the cutaneous withdrawal re ex, features of the response evoked preferentially or specifically by noxious stimuli in the adult can be elicited by non-noxious stimuli in the neonate. Apart from this question of pain perception, there is now increasing evidence that early exposure to noxious stimuli results in adverse effects on future neural development. That is to say, noxious stimulation might not need to reach consciousness to substantially alter the course of sensory development and postnatal outcome for the individual.

In summary, the essential anatomical substrates for transmitting somatosensory input to the cerebral cortex appear to be established prior to birth in humans, in the sheep, and presumably also in other species that undergo considerable development in utero during a long gestation period. Of course, this information still does not provide an obvious answer to the question as to when stimulation of the body can first be perceived by a fetus or neonate. But once the essential projections are present and have matured in the cortex there may be no obvious critical stage or defining point in development that results in a step from nonperception to perception of a stimulus. It seems more likely that these properties emerge gradually and mature over a long period of time in parallel with the maturation of cortical functions as a whole.

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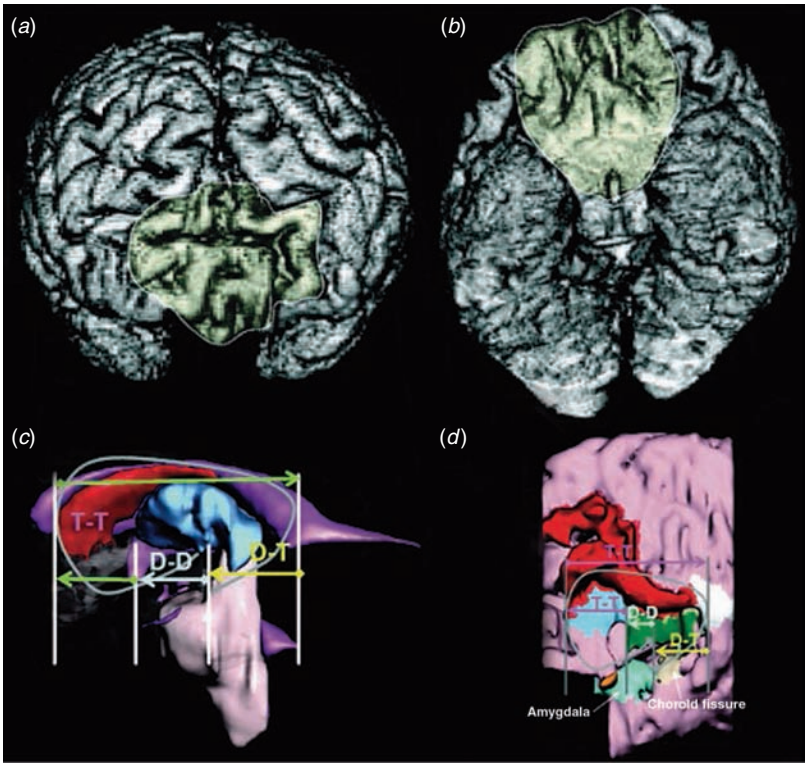

Fig. 3.2 Anatomic overview of holoprosencephaly (HPE). (*a*) Frontal and (*b*) inferior views. A veil is superposed over the rostral conjunction of the left and right cortices with no intervening interhemispheric fissure, which emerges immediately caudal to the veil. (*c*) Topological segments: D-D, the diencephalic segment extends from the subthalamus to the suprachiasmatic junction with the telencephalon. T-T, the telencephalic segment, extends from the D-D segment through the septal limb of hemisphere to the hippocampal commissure. D-T, the diencephalic-telencephalic segment, diverges laterally from the midline to follow the temporal limb of the choroid fissure to the amygdala. (*d*) The seam and adjacent structures as seen from the midsagittal aspect in HPE (Takahashi *et al*., 2003). (With permission of Oxford University Press.)

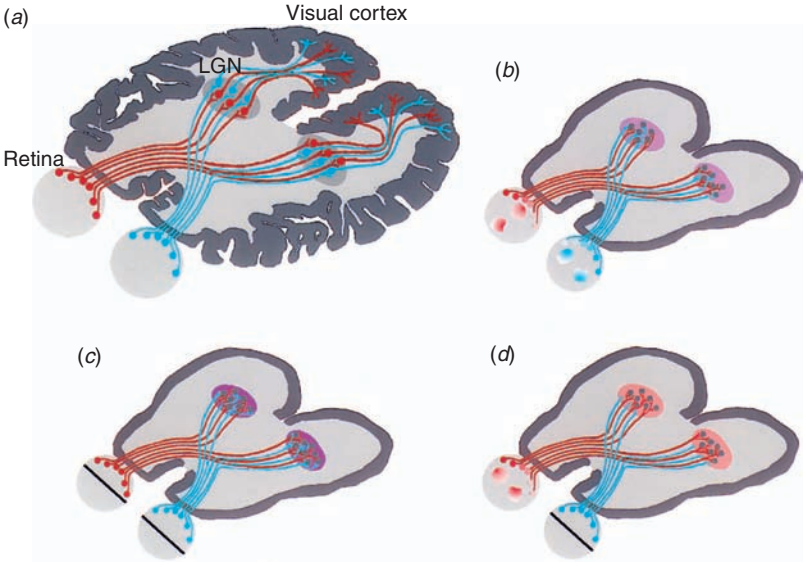

Fig. 10.1 (*a*) Specificity of the adult mammalian visual pathway. Retinal ganglion cell (RGC) axons project to the LGN topographically. The nasal axons cross over at the optic chiasm to the contralateral side of the brain, whereas the temporal axons project to the lateral geniculate nucleus (LGN) on their own side of the brain. In the LGN, the RGC axons terminate in discrete eye-specific layers (only two are shown schematically here). The LGN neurons in turn project to the primary visual cortex, maintaining the retinotopic map. The LGN axons terminate in layer IV of the primary visual cortex where they are segregated into alternating eye-specific regions that form the basis of the ocular dominance column (ODC). (*b*) Developing visual pathway. During development, the projection from the retina to the LGN is not segregated into eye-specific layers. The retinal axons from the two eyes initially overlap substantially (only the binocular portion of the LGN is shown). Refinement of this projection into the precise, mature pattern of connections requires neural activity (Shatz & Stryker, 1988). During this process of segregation, waves of spontaneous, correlated activity that vary in location and direction spread through the developing retina (shown schematically in the eyes as colored patches representing the activity of groups of RGCs; the gradation in intensity from dark to light indicates the direction of propagation over time) (Feller *et al*., 1996). After the layers in the LGN have formed, the LGN neuron axons will segregate from an initially diffuse projection into the ODC of the visual cortex (not shown). (*c*, *d*) Disruption or imbalance of retinal activity blocks the development of the visual pathway. (*c*) When the RGC waves of activity are blocked during RGC axon segregation, the layers in the binocular region of the LGN fail to form. When the RGC activity in both eyes is blocked, segregation does not occur; both sets of axons expand the LGN territory that they fill. (*d*) When the balance of competition is disrupted by blocking the activity in only one eye, the active eye (red) gains most of the binocular territory in the LGN and many of the axons from the inactive eye (blue) are driven out (Penn *et al*., 1998).

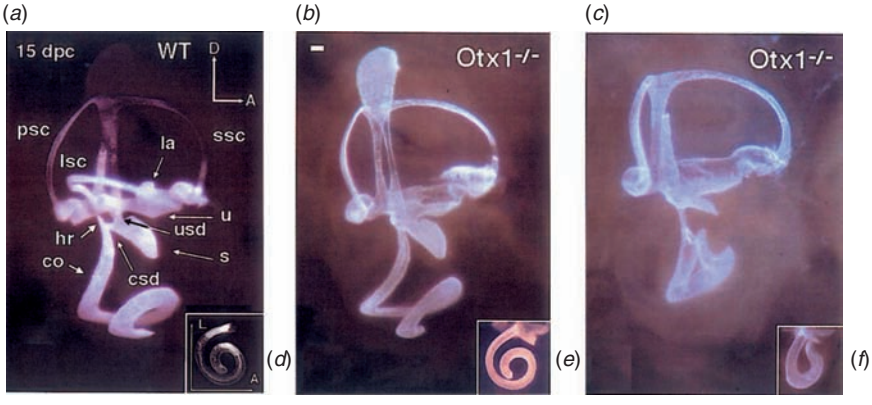

Fig. 11.8 Paint-filled inner ears of wild-type (wt) and *Pax2* mutants at 15.5 dpc. (*a*), (*b*), and (*c*) are lateral views and (*d*), (*e*), and (*f*) are anterior views of wt or mutant inner ears. (*a*, *d*) A wt inner ear showing a fairly mature morphology. (*b*, *e*) A *Pax2* homozygous inner ear with a less severe phenotype than the one in (*c*) and (*f*). The cochlea is shortened, and the common crus and the endolymphatic duct (ed) are fused. The semicircular canals are slightly thinner than wt. (*c*, *f*) A more severe form of a *Pax2* mutant inner ear with semicircular canals displaced laterally due to exencephaly. The diameter of the semicircular canals is larger than wt, and the cochlea is shortened. However, the fusion between the common crus and the ed is at a more ventral location than the specimen shown in (*e*). lsc, lateral semicircular canal; psc, posterior semicircular canal; s, saccule; u, utricle. Orientation on arrows: A, anterior; D, dorsal; L, lateral. (From Burton *et al*., 2004 with permission.)

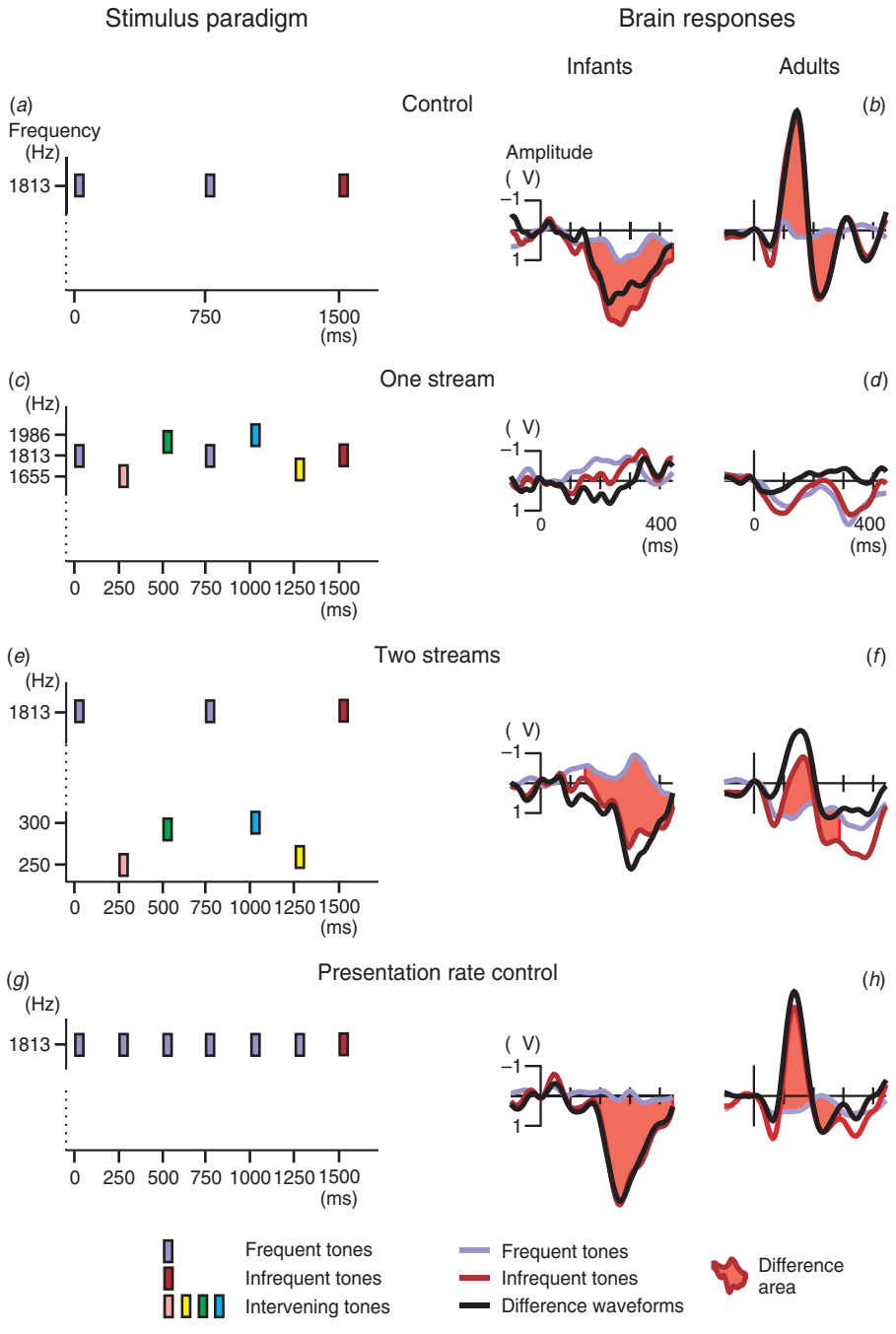

Fig. 12.2 Sound streams segregation in newborns and adults: the stimulus paradigm and electric brain responses.

(*a*) Schematic illustration of a segment of the control-condition sequence. Rectangles represent tones; their y-axis coordinate shows the tone frequency (logarithmic scale). Different loudness-level settings are marked with different colors: pastel blue, frequent soft (standard) tones; dark red, infrequent louder (deviant) tones. (*b*) Control-condition responses. Frontal (F4) electric brain responses (*left*, infants; *right*, adults) elicited by the standard (pastel blue lines) and deviant (dark red line) tones together with their respective difference waveform (black line). The light red shading of the area between the standard and deviant responses marks significant differences between the two brain responses. (*c*) In the one-stream condition, intervening tones varied in frequency (shown by the y-axis position of the rectangle) and intensity (marked with different colors). (*d*) One-stream condition responses. The responses to the standard and deviant tones did not significantly differ from each other in either group of subjects. (*e*) For the two-stream condition, the frequencies of the intervening tones were lowered from the values used in the one-stream condition (see y-axis positions), but the tone intensity values were retained (see rectangle colors). (*f*) Two-stream condition responses. The responses to the standard and deviant tones significantly differed from each other in both groups of subjects, and they were similar to those elicited in the control condition. (*g*) In the presentation-rate control experiment, "intervening" tones were identical to the frequent tones (same frequency and same intensity). (*h*) Presentation-rate control experiment responses. The responses to the standard and deviant tones significantly differed from each other in both groups of subjects and were similar to those elicited in the control and two-stream conditions of the main experiment. Note the positive polarity of the newborns' mismatch response in this study. (Reprinted with permission from Winkler *et al*. (2003), © 2003 *National Academy of Sciences of the U S A*.)

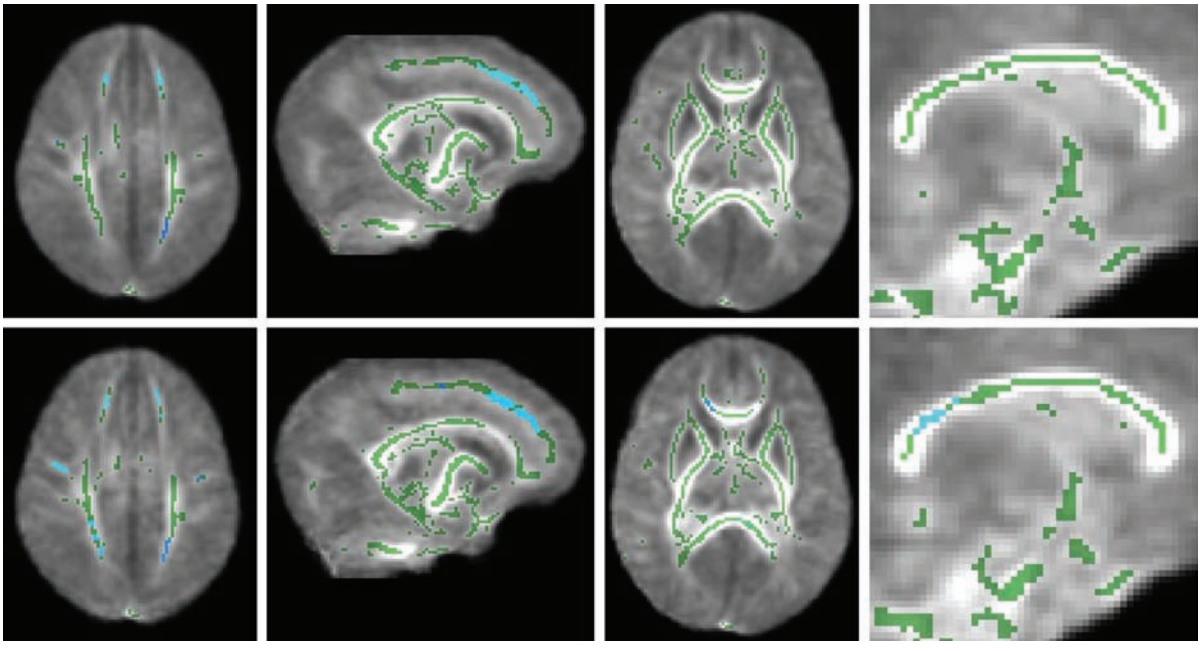

Fig. 13.11 Tract-based spatial statistics (TBSS). Using diffusion tensor imaging, an atlas of white matter tracts is derived from values of fractional anisotropy (FA). The top row compares the FA between 26 preterm infants at term-equivalent age against six term-born controls. Regions in green showed no difference in FA between the groups, whereas regions in blue showed a significantly lower FA in the preterm group, correcting for multiple comparisons using cluster-based thresholding. The bottom row shows the comparison between 11 preterm infants at term that were born ≤28 weeks' gestation compared with the six term controls. Regions in blue indicate a significantly lower FA in the preterm group (Anjari *et al.*, 2007).

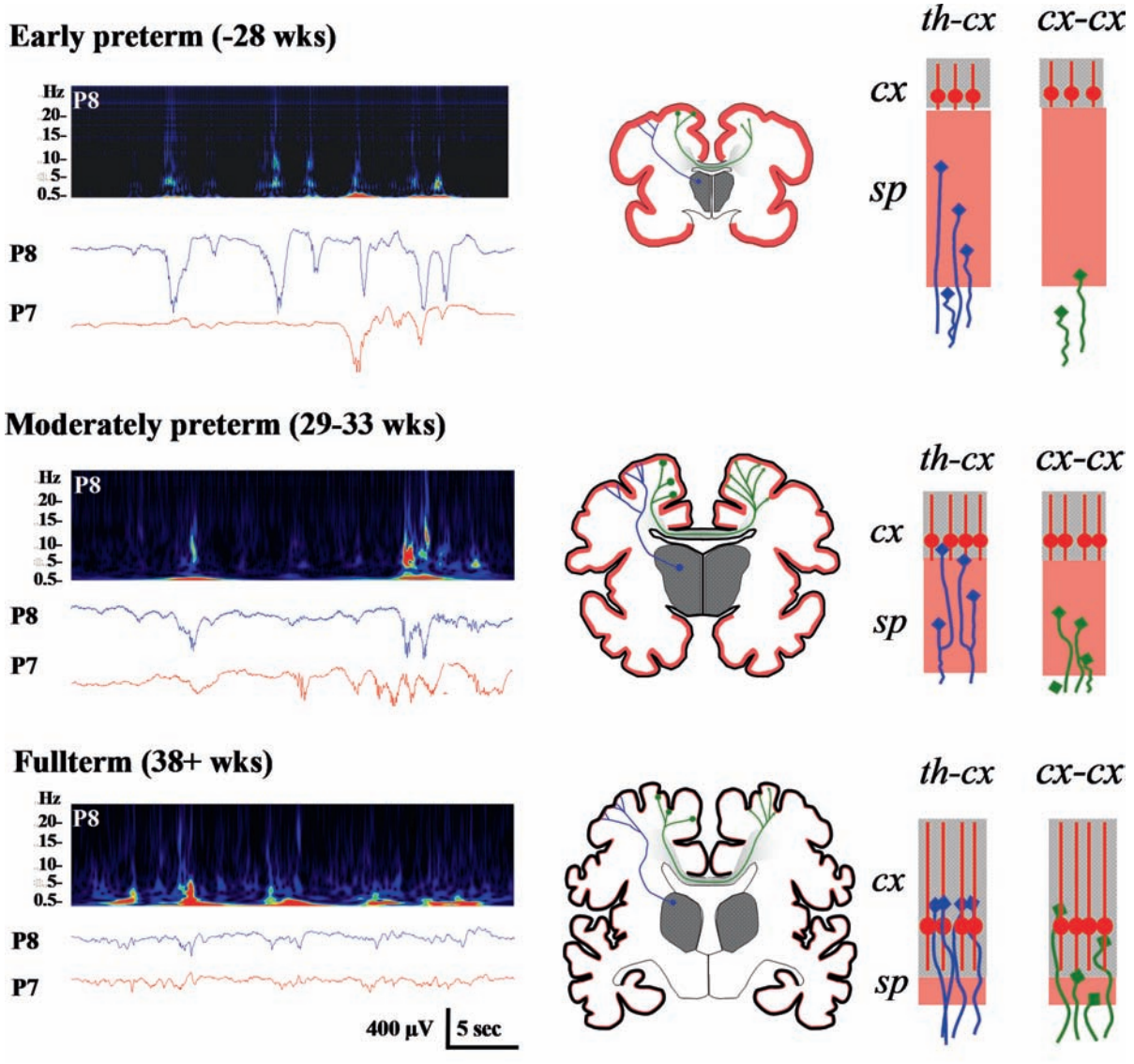

Fig. 15.1 Major developmental milestones of cortical function (left side) and structure (right side) of the human preterm baby. Left side shows one EEG trace (unpublished data) from each parietal area lobe (P8 is right and P7 is left; Fz reference). In addition, a time-frequency representation of the P8 trace is shown to demonstrate the multiband nature of the EEG events (see main text). In the early preterm baby, the EEG activity consists mainly of brief, monophasic, high-amplitude events, which are relatively independent in each cortical area. The thalamocortical (th-cx) connections are just arriving at the subplate–cortex layers, whereas the corticocortical (cx-cx) connections have not yet grown. In the moderately preterm baby, the EEG activity consists of a little longer but still rather high-amplitude, mono- or biphasic events, which gradually become more synchronized between distant cortical sites. The thalamocortical connections are now being established in the cortex proper, and the corticocortical connections are growing into the subplate and deep cortical layers of the target areas. In the full-term baby, the EEG activity consists of relatively long and lower amplitude events, which are relatively synchronized within and between the hemispheres. In addition, there is abundant ongoing (continuous) activity during the intervals between the discrete events. The thalamocortical connections are now mostly established in the cortical layer IV, and the corticocortical connections are innervating their final target zones and layers in the target areas.

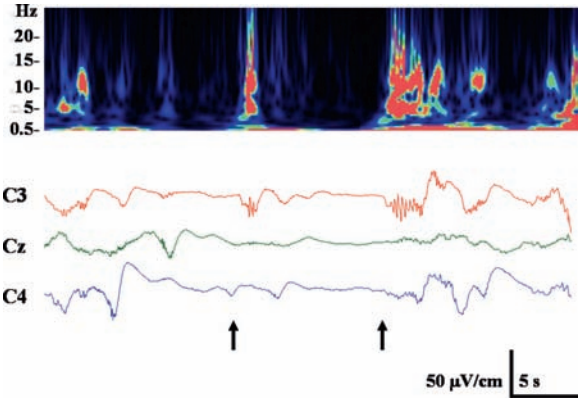

Fig. 15.2 Cortical somatosensory response in a preterm baby (conceptional age, 30 weeks). Tactile (brush) stimulation of the right palm leads to a long-lasting (one to three seconds) activity transient within the somatosensory cortex of the contralateral (left; C3) hemisphere, whereas no comparable activation is seen in the more medial or ipsilateral cortical areas (Cz and C4, respectively). In contrast, all these cortical areas exhibit SAT events, which in the time-frequency (wavelet) representation look remarkably similar to the "evoked activity." EEG traces (unpublished data) are shown in a current source density (Laplacian) montage.

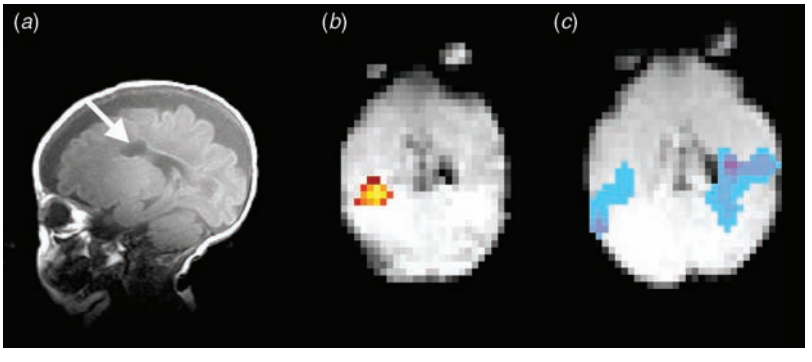

Fig. 20.2 Evidence that the functional magnetic resonance imaging (fMRI) BOLD response to auditory activation can change over time in a preterm infant with grade 4 intraventricular hemorrhage (IVH). This 24-week gestational age preterm infant with a left-sided grade 4 IVH and subsequent porencephaly (*a*; arrow) was first studied with echoplanar fMRI using a frequently modulated pure tone at term equivalent age; only right-sided BOLD signal in response to stimulus was detected (*b*) at that time. Six weeks later (*c*), bilateral auditory activation was detected using the same experimental paradigm. (Courtesy of A. Anderson, Vanderbilt University.)

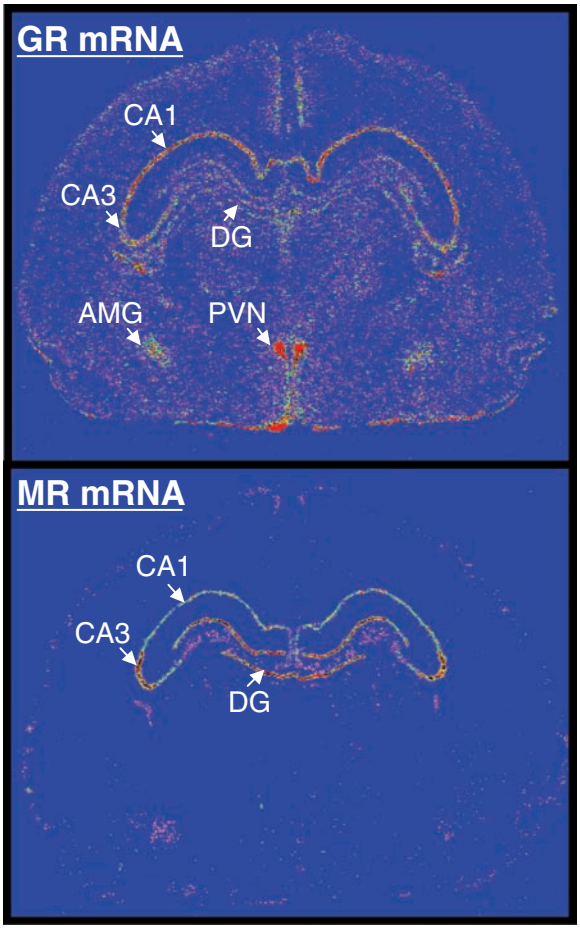

Fig. 22.2 Color-enhanced image illustrating the expression of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA in coronal sections of the fetal guinea pig brain. Receptor mRNA was determined by *in situ* hybridization using ^{35}S-labeled oligonucleotide probes specific for GR and MR. GR mRNA is present at high levels in the CA1–CA4 fields of the hippocampus, the dentate gyrus (DG), paraventricular nucleus (PVN) and amygdala (AMG). Lower levels of GR mRNA are present in other brain regions. In contrast, MR mRNA is confined almost exclusively to the limbic system. Red, high expression; yellow, moderate expression; green, low expression; blue, no expression.

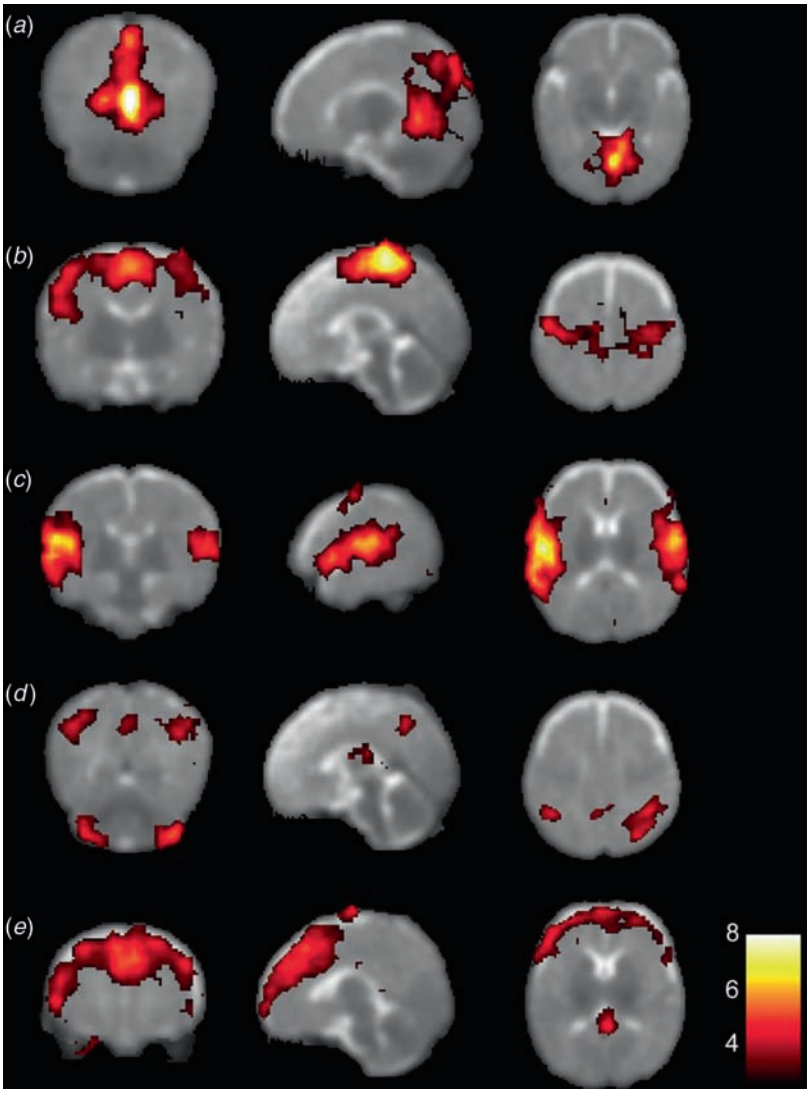

Fig. 23.3 Functional magnetic resonance imaging used to map the resting state activity of the brain in lightly sedated infants around term reveals several unique resting-state networks that encompass (*a*) the primary visual cortex, (*b*) the somatosensory and motor areas, (*c*) primary auditory cortex, (*d*) posterior lateral and midline parts of the parietal lobe and lateral aspects of cerebellum, and (*e*) the medial and dorsolateral prefrontal cortex, a network potentially of importance for access to consciousness. (From Fransson *et al*., 2007.)

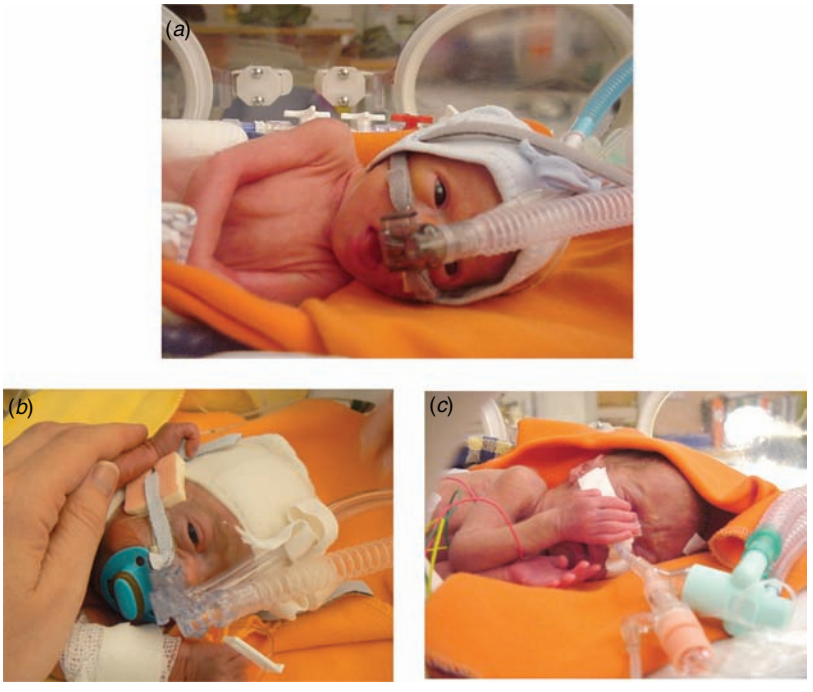

Fig. 23.5 Preterm infants of about 26 weeks are awake for short periods and seem to fix the gaze for short moments toward the mother. They also seem to be able to move their arms for protection (self-awareness). (Photos courtesy of Ann-Sofi Gustafsson.)

Chapter 10

Principles of endogenous and sensory activity-dependent brain development: the visual system

Anna A. Penn and Carla J. Shatz

Activity-dependent remodeling of early neural connections

As the human brain develops, billions of neurons cause an average of 1000 synapses to become interconnected in precise neural circuits. How are these complex neural connections established? Although many details are still poorly understood, the required steps are now understood in general outline. First, cells must be generated by successive cell divisions and their identity must be determined – as neurons, and then as particular classes of neurons. Second, neurons from one region must extend axons along specific pathways to appropriate target regions to form the linked pieces of a functional system. However, the initial pattern of connections is often imprecise. The third step is the refinement of these connections to form the specific patterns of connectivity that characterize the mature brain.

For the past half-century, the mammalian visual system has been the model of choice in which to examine the formation of precise neural connections. Neural activity in the visual systems is critical for sculpting its intricate circuits from initially imprecise connections. The necessity for normal visual input has been widely recognized as crucial for the developing brain, with loss of normal input leading to profound, irreversible changes in visual function. New experiments demonstrate a similar requirement for endogenous neural activity generated by the nervous system itself, long before the onset of vision. In this chapter, it is this last step of circuit formation – refinement controlled by neural activity – that will be discussed. The visual system is used here as a specific example, but the same general processes have been described in other brain areas where precise maps form from initial coarse connections (Fox & Wong, 2005; Luo & Flanagan, 2007). Since many brain regions use the same types of mechanisms, it is likely that disruptions in early neural activity, and thus disruptions in the formation of precise circuits, may underlie many common neurodevelopmental disorders, ranging from subtle learning disorders to pervasive developmental delay. Understanding the processes of activity-dependent development should contribute substantially to our future ability to identify, prevent, and treat many neurodevelopmental disorders that result from disruptions of neural activity during critical periods in brain development.

The first two steps of circuit formation – cell type determination and pathway formation – are often referred to as "activity-independent" processes because, in general, neuronal activity (i.e., transmission between presynaptic and postsynaptic partners via action potentials and neurotransmitter release) is not required for these processes to occur. Rather, signals for di erentiation, cell type determination, and axonal guidance are given by genetically specified molecular cues that appear to provide for the reliable construction of a stereotyped framework (Goodman & Shatz, 1993; O'Leary & McLaughlin, 2005). Most clinical work has focused on neurogenetics and the role of activity-independent factors in pediatric neurological disease. Numerous "activity-independent" neurological diseases have been described: disorders of neural induction (anencephaly); disorders of neurogenesis (as seen in the "minibrain" mutation); disorders of programmed cell death (spinal muscular atrophy); disorders of neural migration (focal dysplasias or heterotopias); and disorders of axonal pathfinding (agenesis of the corpus callosum).

The Newborn Brain: Neuroscience and Clinical Applications, 2nd edn., eds. Hugo Lagercrantz, M. A. Hanson, Laura R. Ment, and Donald M. Peebles. Published by Cambridge University Press.

In contrast, the elaboration, retraction, and remodeling of neural connections within their targets is thought to be activity dependent because the ultimate patterns of connections can be disrupted by blockade of neuronal activity (Goodman & Shatz, 1993; Katz & Shatz, 1996; Torborg & Feller, 2005). However, the list of activity-dependent disorders has been much shorter. Traditional histological and radiological studies in human subjects can visualize only the most profound disruptions in connectivity – those that result in major structural defects. Yet manipulations that disrupt normal neural activity can cause large changes in the detailed patterns of synaptic connections while leaving gross brain structure intact (Shatz & Stryker, 1988). In addition, the activity-independent disorders mentioned above often result in abnormal neuronal circuitry, making primary and secondary causes of neurological diseases di cult to untangle. In the past few years, noninvasive functional imaging techniques have opened up exploration of functional changes in neural circuitry during development. Autism and schizophrenia are being investigated as possible examples of errors in activity-dependent circuit development (Penn, 2001; Rapoport *et al.*, 2005; Minshew & Williams, 2007; Uhlhaas & Singer, 2007). Formulating specific hypotheses regarding the mechanisms that may underlie activity and circuit abnormalities in these disorders and identifying more potential "activity-dependent" disorders depends first on understanding the general principles of activity-dependent development, as exemplified by the visual system.

The developing visual pathway as a model system

The development of eye-specific connections in the mammalian visual pathway is a model system for illustrating the principles of activity-dependent synaptic refinement: the precise anatomy of this pathway is well defined; the need for neural activity in the formation of visual connectivity has been documented; specific patterns of neural activity present in this pathway have been described; and the mechanisms of synaptic reorganization underlying the fine-tuning of these connections are being investigated intensively.

Anatomy of the system

In the mammalian visual system, retinal ganglion cell (RGC) axons from each eye synapse onto cells in the lateral geniculate nucleus (LGN). The LGN neurons then project to layer IV of the visual cortex (Fig. 10.1*a* in color plate section). A striking aspect of this pathway is a precise retinotopic map in each region as well as segregation of the inputs coming from each eye. The axons from nasally placed ganglion cells project to contralateral LGN and axons from temporally placed ganglion cells project to the ipsilateral LGN, whereas the nearest neighbor relations of the RGCs are preserved, projecting a retinotopic map of one hemisphere of the visual world onto the opposite LGN. Within the LGN, the axons from the two eyes terminate in a set of separate, alternating layers in which LGN neurons receive purely monocular inputs. In turn, the LGN projection to layer IV of the primary visual cortex again retains the retinotopic map. In addition, the patterns of connections form ocular dominance columns (ODCs), alternating 0.5-mm-wide columns of cortical neurons driven only by input from one eye, and form orientation columns (or "pinwheels") – groupings of neurons driven by stimuli of the same orientation (reviewed in Rodieck, 1979). Even in mice, which have minimal binocular vision, inputs are segregated in an eye-specific manner in their small binocular region of the cortex (Jaubert-Miazza *et al.*, 2005). RGCs also project to the superior colliculus, and the axon terminals are again grouped in an eye-specific and cell-class-specific manner (Stein, 1984).

In the initial development of the visual system, neither the layers in the LGN nor the cortical ODCs are present, and the retinotopic map is crude. When RGC axons from each eye first innervate the LGN, they are intermingled in most of the nucleus (Fig. 10.1*b*) (Sretavan & Shatz, 1986). The LGN neurons receive binocular innervation during this period (Shatz & Kirkwood, 1984), but, through a process of axon retraction and elaboration, connections are refined and the adult pattern emerges. Similarly, the LGN projections to layer IV are initially intermingled. LGN axons representing either the right or left eye then segregate to define ODCs in the primary visual cortex (LeVay *et al.*, 1978) or eye-specific inputs to single cortical neurons in the mouse (Jaubert-Miazza *et al.*, 2005). Although the precise timing of ODC development in various species has been debated in recent years (Katz & Crowley, 2002), there is no doubt that connections are initially imprecise and that the eye-specific segregation of axons in the LGN always precedes eye-specific LGN innervation of cortical neurons.

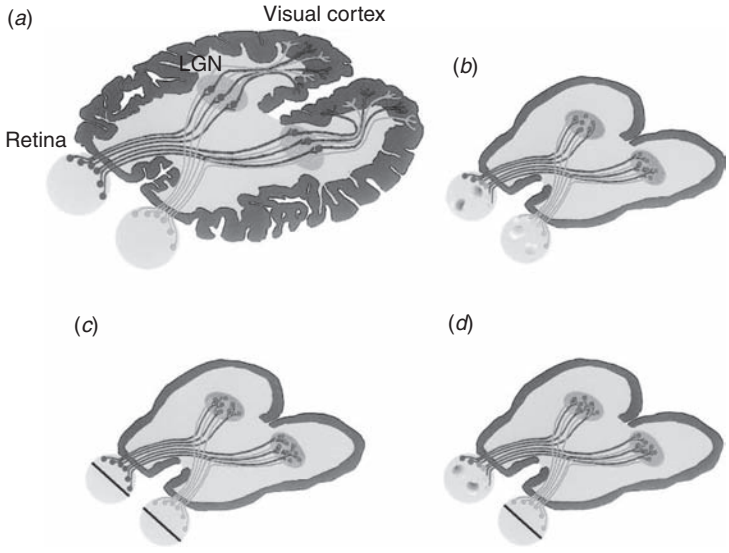

Fig. 10.1 (See color plate section.) (*a*) Specificity of the adult mammalian visual pathway. Retinal ganglion cell (RGC) axons project to the LGN topographically. The nasal axons cross over at the optic chiasm to the contralateral side of the brain, whereas the temporal axons project to the lateral geniculate nucleus (LGN) on their own side of the brain. In the LGN, the RGC axons terminate in discrete eye-specific layers (only two are shown schematically here). The LGN neurons in turn project to the primary visual cortex, maintaining the retinotopic map. The LGN axons terminate in layer IV of the primary visual cortex where they are segregated into alternating eye-specific regions that form the basis of the ocular dominance column (ODC). (*b*) Developing visual pathway. During development, the projection from the retina to the LGN is not segregated into eye-specific layers. The retinal axons from the two eyes initially overlap substantially (only the binocular portion of the LGN is shown). Refinement of this projection into the precise, mature pattern of connections requires neural activity (Shatz & Stryker, 1988). During this process of segregation, waves of spontaneous, correlated activity that vary in location and direction spread through the developing retina (shown schematically in the eyes as colored patches representing the activity of groups of RGCs; the gradation in intensity from dark to light indicates the direction of propagation over time) (Feller *et al.*, 1996). After the layers in the LGN have formed, the LGN neuron axons will segregate from an initially diffuse projection into the ODC of the visual cortex (not shown). (*c*, *d*) Disruption or imbalance of retinal activity blocks the development of the visual pathway. (*c*) When the RGC waves of activity are blocked during RGC axon segregation, the layers in the binocular region of the LGN fail to form. When the RGC activity in both eyes is blocked, segregation does not occur; both sets of axons expand the LGN territory that they fill. (*d*) When the balance of competition is disrupted by blocking the activity in only one eye, the active eye (red) gains most of the binocular territory in the LGN and many of the axons from the inactive eye (blue) are driven out (Penn *et al.*, 1998).

General principles of activity-dependent development

Molecular cues may have an important role in the initial spatial pattern of axonal connections (O'Leary & McLaughlin, 2005), at least at the level of the LGN and colliculus, but neural activity is required for fine-tuning of eye-specific axon segregation in both the LGN and cortex (reviewed in Firth *et al.*, 2005; Huberman, 2007).

Competition shapes the patterns of connections

Hubel and Wiesel's seminal experiments in the 1960s launched the study of neural competition in brain development (Hubel & Wiesel, 1965, 1970). There is now strong evidence that the fine-tuning of axonal connections is achieved by an activity-mediated *competitive* process involving the formation and elimination of specific synapses. The process is competitive in the sense that unequal levels of neuronal firing, or asynchronous use of synapses, results in the dominance of connections from the more active eye at the expense of those from the less active eye (Figs. 10.1*b*, *d* and 10.2). Synapses that can be remodeled by activity are likely to have characteristics of hebbian synapses – defined as synapses that are strengthened by the synchronous firing of both presynaptic and postsynaptic neurons (Hebb, 1949). In other words "cells that fire together, wire together" and, as a corollary, "those that don't, won't." When this process is disrupted by unequal activity levels in the two sets of inputs, the active inputs have a competitive advantage and gain more connections.

Many experiments show that the balance of activity is crucial to the refinement of visual neuron connections (reviewed in Katz & Shatz, 1996). As Hubel and

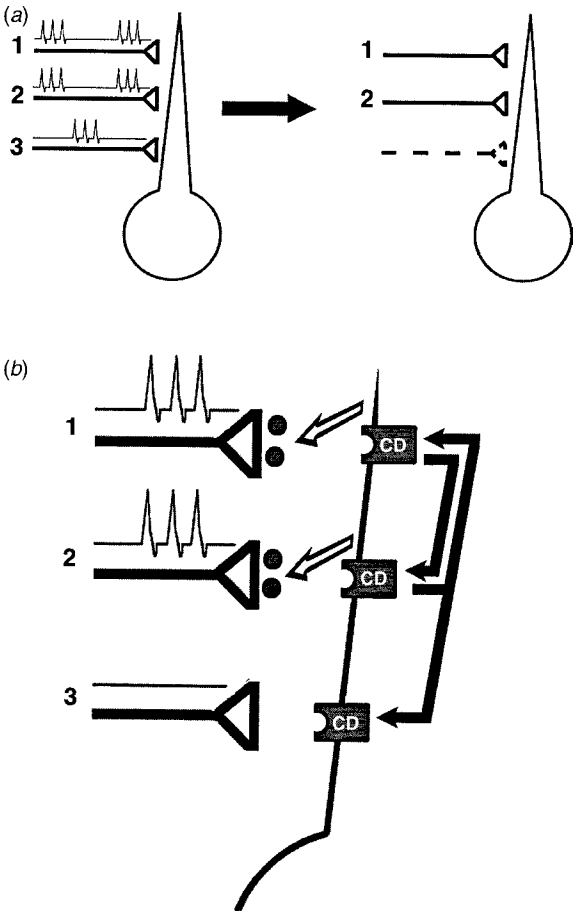

Fig. 10.2 (*a*) Synaptic modifications based on patterns of activity. When coinnervating axons are synchronously active (axons 1 and 2; illustrated by the pattern of action potentials drawn schematically above each axon) with the postsynaptic neuron, they will both be maintained and strengthened. However, when a coinnervating axon is asynchronously active (axon 3), then this axonal connection will be lost. (*b*) Predicted signals for the activity-dependent refinement of connections. An enlargement of the schematic shown in (*a*) illustrates the basic mechanisms that are necessary for activity-dependent competition. When axons 1 and 2 fire action potentials synchronously and depolarize the postsynaptic neuron, the postsynaptic neuron must have a mechanism for detecting this coincident firing (a coincidence detector [*CD*], such as an *N*-methyl-D-aspartate [NMDA] receptor). When presynaptic axons are simultaneously active, a "retraction" signal is produced (black arrows). The active axons (1 and 2) are protected from elimination, perhaps by a retrograde messenger that specifically stabilizes only active synapses (open arrows). When the inactive axon receives the retraction signal (axon 3), it is not protected and will therefore be eliminated. When all of the axons are inactive (not shown), no signals will be sent and the connection will remain (although they may be weaker than normal because of lack of support from retrograde messengers, such as neurotrophins; see text). Note that the messengers that govern protection and retraction may be presynaptically or postsynaptically generated (they are shown as postsynaptic here only for simplicity).

Wiesel first observed, closing one eye in a kitten during its early postnatal development profoundly disrupted the pattern of ODCs; the eye that had visual input dominated the cortex, whereas the closed eye lost its connections. Cortical connections to the opened or closed eye, assessed both physiologically and anatomically, can be changed in less than a single week of deprivation. When both eyes are closed and consequently there is no imbalance in ocular activity, the ODCs can still form, but much more slowly. Visual deprivation does not abolish spontaneous RGC activity and eye-specific correlation of the remaining retinal activity may drive this segregation. ODC segregation can be blocked by eliminating all ascending activity in the visual pathway, achieved by silencing RGC activity intraocularly with the sodium-channel blocker tetrodotoxin (TTX). If the optic nerves are then stimulated electrically, the normal shift from initial binocular innervation of cortical neurons to the emergence of monocularly driven cells can be achieved, indicating that neural activity drives this process. However, electrical stimulation only induces ocular segregation at the cortical level when it is applied to the two optic nerves in an alternating (asynchronous) manner. If the optic nerves are stimulated synchronously, cortical neurons remain binocularly innervated and segregation does not occur, again suggesting that axonal competition is critical.

Additional evidence for the role of neural competition in generating precise connections comes from studies of cold-blooded vertebrates (reviewed in Cline, 1991). Neural activity is required for topographical map development in the tectum (the equivalent of the superior colliculus in mammals) and for the segregation of eye-specific stripes that can be experimentally induced in frog optic tectum. (The "three-eyed" frog model is produced by grafting a third eye that sends its RGC axons to the tectum, normally monocular in frogs, such that the axons from the two eyes now innervating a single tectum segregate into eye-specific stripes.) Blockade of activity prevents refinement of the retinotectal map from an initially coarse projection and desegregates the eye-specific stripes in the tectum of the three-eyed frog, which require activity to be maintained even after they have formed because the frog tectum continues to grow throughout life. Again, experiments show that it is the temporal pattern of neural activity that is critical for the process of axonal refinement. Rearing goldfish or three-eyed frogs under strobe lights, which synchronizes the

activity of all ganglion cells, prevents topographical map refinement in the fish and eye-specific stripe formation in frogs. Taken together, these observations demonstrate that not just activity per se, but specific aspects of the timing of electrical activity, is necessary for this axonal segregation. Synapse formation on the basis of spatiotemporally correlated activity may be a general mechanism used throughout the vertebrate nervous system to establish precise connections (Goodman & Shatz, 1993; Katz & Shatz, 1996).

Critical periods for competition

The time when normal patterns of neural activity are necessary for the formation of the adult pattern of connections has been called the "critical period" for the development of those connections. In humans, one example of activity-driven rearrangements occurring during a critical period is the loss of functional vision (e.g., amblyopia) in one eye that occurs when a child younger than 18 months with a cataract goes untreated for more than one to two weeks (Lewis & Maurer, 2005). Even temporary disruption of appropriate sensory input can permanently a ect cortical patterns of connections if it occurs during the appropriate critical period, as Hubel and Wiesel's studies of monocular visual deprivation in kittens first demonstrated. The plasticity of connections inherent in the critical period is followed by relative stability in the mature pattern of connections. This stability of adult neural connections is seen when a cataract forms in an adult. In contrast to the infant with a cataract, normal vision is restored in the adult who has the cataract removed, even if it has been present for many years, because the neural connections in the mature visual pathway do not change. In the past decade, our understanding of "critical periods" has become more nuanced with the recognition that there may be multiple periods of *sensitivity* to changes in neuronal activity, periods that encompass not only the initial refinement of connections but also the stabilization and maintenance of these connections (reviewed in Lewis & Maurer, 2005; Huberman, 2007). The signals and mechanisms that control these periods of plasticity are being actively studied.

The synaptic plasticity present in the developing nervous system endows it with the ability to adapt to the many variations of the external world, as in language acquisition, or the ability to recover from early damage when one region of cortex subsumes the functions of a damaged area. However, this plasticity also leaves the developing nervous system uniquely vulnerable to injury from abnormal patterns of activity. In the human, this period of neuronal vulnerability extends from the end of the second trimester of gestation well into childhood.

Neuronal activity: spontaneous and sensory driven

Requirement for spontaneously generated activity

How early in development is neural activity needed for the refinement of connections? Before the development of vision – even before retinal photoreceptors develop – RGCs fire spontaneous action potentials and this activity is transmitted through the entire developing visual pathway (reviewed in Firth *et al.*, 2005). The hypothesis that this spontaneous activity drives eye-specific refinement in the LGN and formation of visual cortex ODCs has been much investigated and hotly debated in the past few years (see Katz & Crowley, 2002; Firth *et al.*, 2005; Chalupa, 2007; Huberman, 2007).

Recall that before ODC form, RGC axons segregate to form the eye-specific layers in the LGN (Fig. 10.1*b*). This process occurs entirely during the period before photoreceptors mature, yet it depends on neural activity. Infusion of TTX into the LGN, which blocks both presynaptic and postsynaptic sodium action potentials, blocks this layer formation (Shatz & Stryker, 1988). Individual axons in TTX-treated LGN branch widely, rather than retracting inappropriate branches and growing selectively into their appropriate eye-specific layers (Sretavan *et al.*, 1988). These initial experiments pointed to a need for neural activity before vision, activity that was discovered to be generated by the developing retina (reviewed in Firth *et al.*, 2005).

To test the role of this spontaneous retinal activity, we selectively blocked only the RGC activity in the retina, rather than the activity in the LGN (Penn *et al.*, 1998). These were the first experiments to show that spontaneous retinal activity is required for axon segregation in the LGN and that activity di erences leading to competition between the two eyes is critical. When activity was blocked binocularly, layers also failed to form, indicating a requirement for the presynaptic action potentials (Fig. 10.1*c*). But when the

spontaneous retinal activity was blocked only in one eye, the projection from the active retina expanded greatly into territory normally belonging to the other eye, and the projection from the inactive retina was substantially reduced (Fig. 10.1*d*). Thus, interocular competition – driven exclusively by endogenous retinal activity – determines the pattern of connections. These experiments demonstrate that spontaneous activity can produce highly stereotyped patterns of connections long before the onset of visual experience. However, these experiments do not address whether it is general neural activity or a particular pattern of activity that is needed. In the past decade, new techniques in recording from and imaging live neural tissues have deepened our understanding of the spatiotemporal parameters and mechanisms of spontaneous activity, allowing direct testing of the need for particular activity patterns.

Patterns of spontaneous activity

Many areas of the nervous system generate complex patterns of activity long before there is any patterned sensory input (Feller, 1999). Is it the activity or the patterns of this activity – the spatiotemporal correlations – that are required for activity-dependent competition? To answer this question, the patterns themselves must be defined and then their role in shaping neural connection needs to be tested. This distinction between needing *any* activity and needing *spatiotemporally correlated activity* has profound implications for the types of neural mechanism that might be active, as well as for the types of process that might interfere with normal patterning.

Ex utero microelectrode recordings from fetal rat retinas first indicated that cells in the ganglion cell layer are spontaneously active and fire together long before there is any visual input (Ma ei & Galli-Resta, 1990). More recently, simultaneous recordings from hundreds of neurons in developing retinas in vitro, using either a multielectrode array or optical recording techniques (Meister *et al.*, 1991; Feller *et al.*, 1996), reveal a particular spatiotemporal pattern of firing. Individual ganglion cells fire bursts of action potentials of two to eight seconds in duration, separated by extended periods of quiescence 40–90 seconds long (Wong *et al.*, 1993). Measurements from groups of cells show that neighboring cells fire action potentials together and undergo increases in levels of intracellular calcium synchronously (Meister *et al.*, 1991; Feller *et al.*, 1996). On a larger spatial scale, the pattern of activity resembles a "wave" that travels across local regions of the retina at about 100–300 μm/s, and involves cells situated within approximately 300 μm of each other (Wong *et al.*, 1993). These waves "tile" the retina with highly restricted domains of activity whose borders change over time (Feller *et al.*, 1996). Importantly, the retinal waves are only present during the period of retinogeniculate axon terminal segregation and are gone by the time photoreceptors are present and visual input is available. After this early period, RGCs are spontaneously active in the dark, but do not participate in waves of activity (Wong *et al.*, 1993). Similar spontaneous, correlated retinal activity is present in mammals (cat, ferret, and mouse as well as in turtle and chick as reviewed in Feller, 1999), emphasizing that it may be a well-conserved mechanism for refining neural connectivity before vision.

The early pattern of spontaneous retinal activity is well suited to drive segregation not only because it is present during the appropriate period, but also because of these particular spatiotemporal characteristics. The short duration of the activity compared with the long intervening periods of silence makes it unlikely that presynaptic cells from the two eyes will be active simultaneously (Fig. 10.2*a*). Spurious correlations are unlikely because the location, timing, and direction of wave spread are highly variable within a single piece of retina (Meister *et al.*, 1991; Wong *et al.*, 1993; Feller *et al.*, 1996). Within a retina, correlations in the timing of the bursts are stronger between neighboring retinal neurons than for distant neurons. As mentioned earlier, lasting increases in synaptic strength are thought to require presynaptic inputs that are su ciently correlated so that there is an overlap in postsynaptic response. For synaptic weakening, the inputs from the two eyes onto a single postsynaptic neuron should be significantly uncorrelated, resulting in the weakening of one of the two inputs (Fig. 10.2*a*). Thus, the correlations created by the retinal waves could underlie cooperative synaptic strengthening, thought to be necessary to group monocularly driven cells into eye-specific layers in the LGN and, at slightly later ages, into ODCs.

How are these correlated patterns of activity generated? In the retina, experiments point to a major role for synaptic transmission, with contributions from other mechanisms, such as gap junctions (reviewed in Firth *et al.*, 2005). During the period when the waves are generated, the retina contains di erentiated

RGCs and amacrine cells (a class of retinal interneurons). Both of these cell types participate in the waves. A synaptic circuit between RGCs and the cholinergic amacrine cells is necessary for the generation and the propagation of the waves; blockade of cholinergic transmission blocks the waves (Feller *et al.*, 1996; Penn *et al.*, 1998). These results emphasize the extent to which even very immature circuits can produce sophisticated patterns of spontaneous activity.

The generation of spontaneous, correlated activity is not limited to the visual system. Spontaneously generated activity has been described in a variety of locations in the developing central nervous system (CNS), including the cortex, hippocampus, cerebellum, thalamus, superior colliculus, locus ceruleus, spinal cord, and the cochlea (reviewed in Feller, 1999). It varies in pattern, but each pattern provides strong spatiotemporal correlations that may shape synaptogenesis. For example, in immature cortical slices, domains of neurons that tangentially span the thickness of the cortical layers and horizontally are within a 50- to 200-mm circular diameter undergo synchronous calcium increases. Unlike the spontaneous activity in the retina, this pattern of activity appears to be coordinated not by synaptic connections, but rather by gap junctional networks that transmit a chemical signal (inositol 1,4,5-trisphosphate), instead of creating classic electrotonic coupling between the neurons. It has been suggested that these cortical domains of activity help to shape the columnar structure of cortical connections (Katz & Shatz, 1996). The developing spinal cord is also endogenously active and appears to combine the use of synaptic and gap junctional connections to generate patterned activity. Synaptic connections appear to underlie the spontaneous, oscillating bursts of activity that occur in dispersed groups of motor neurons that will innervate either flexor or extensor muscles. There also is extensive gap junctional coupling in the spinal cord between motor neurons that innervate homonymous muscles. The spontaneous, coordinated oscillations of activity seen in developing motor neurons may drive the activity-dependent competition known to refine synaptic connections at the neuromuscular junction (NMJ). The developing retina, cortex, and spinal cord illustrate the variety of neural circuitry – based on immature synapses, gap junctions, or a combination of the two – which can generate the correlated, spontaneous activity that may drive the activity-dependent refinement of connections throughout the developing nervous system.

Spontaneous, highly patterned activity occurs in many areas of the nervous system. Therefore, disruption of particular patterns of endogenous activity could result in abnormalities in neural circuitry. For example, damage could result from prepartum use of drugs that interfere with synaptic transmission (i.e., nicotine) or change gap junctional connections (i.e., alcohols). Large-scale structural brain defects would not be expected to result from these exposures; rather, changes in the patterns of connections might result in subtle deficits, such as the cognitive impairments seen in prenatal nicotine or alcohol exposure (Huizink & Mulder, 2006). Although it is difficult to determine whether such deficits are simply the result of global insults from these agents (i.e., reduced oxygen delivery to the fetus or carbon monoxide poisoning), it is worth considering the specific changes in neural connections that may occur when the patterns of endogenous, correlated activity are disrupted.

Role of patterned spontaneous activity in visual system development

Whether the specific, correlated firing patterns of retinal waves are needed for eye-specific segregation in the LGN or for early formation of ODCs has been the subject of much recent debate (Katz & Crowley, 2002; Firth *et al.*, 2005; Chalupa, 2007; Huberman, 2007). Competition mediated by the hebbian model described above predicts that correlated firing is critical for axon refinement. At first, several experiments have suggested that the correlated retinal "waves" are not as important as first predicted. Despite the decorrelation of retinal waves by depletion of the cells that synchronize their periodicity (Huberman *et al.*, 2003) or by changing the periodicity of the waves (Stellwagen & Shatz, 2002), eye-specific segregation of axons in the LGN remained normal. However, some wave structure remains in these experiments. More recent studies using genetically modified mice indicate that high-frequency (>10 Hz) correlated firing of nearest neighbors and lack of correlation between firing in the two eyes is indeed needed for formation and maintenance of proper connections (Torborg *et al.*, 2005; Demas *et al.*, 2006).

In terms of ODC formation, the debate has been even more fundamental, calling into question the need for any neural activity in the initial segregation of columns (Katz & Crowley, 2002). A reevaluation of the timing of ODC formation showed that initial

eye-specific segregation occurs earlier than previously described, well before the visual input that was thought to segregate the eye-specific cortical columns. But does spontaneous retinal activity play a role here as well? Again, the first attempts to test this requirement concluded that activity was not needed (Crowley & Katz, 2000), but spontaneous activity was blocked after columns start to form. If spontaneous retinal activity is blocked before the ODC formation, normal ODCs never develop and receptive fields are distorted (Huberman *et al.*, 2006). Further studies will be needed to determine if particular aspects of the spatiotemporal patterns of this spontaneous retinal activity instruct axon segregation in the cortex as they appear to do in the LGN itself.

Requirement for sensory-driven activity

Once sensory input becomes available, features of the external environment drive the activity that shapes connections. The shapes and forms present in our visual world provide multiple sources of correlated activity; just the edge of this page of paper can correlate the firing of many RGCs across which the linear image falls simultaneously. Disruptions in these correlations, as discussed earlier, can lead to profound anatomical and functional changes in connectivity within the visual cortex during sensitive periods. Reanalysis of the eye-specific shifts that occur in the cortex in response to visual input manipulation in the context of presensory development of ODCs suggests a role for vision not in ODC formation but in ODC maintenance and plasticity (Lewis & Maurer, 2005; Tagawa *et al.*, 2005). The ability of the developing brain to incorporate information from the external world into its precise circuitry allows it to adapt to a myriad of changing environments.

The use of these two sources of activity – endogenously produced and sensory-driven – should not be viewed as occurring in mutually exclusive periods. New experiments in the visual system demonstrate that spontaneous retinal activity is the driving force for refining LGN connections even after the onset of vision, but that after this early overlap of spontaneous and sensory activity, visual activity is needed to maintain segregation (Hooks & Chen, 2006). If spontaneous activity persists for too long, segregation is disrupted (Demas *et al.*, 2006). It is likewise possible, for example, that childhood seizures undermine normal connectivity by creating spontaneous correlations when none should exist (Holmes, 2005). In other brain regions, early sensory input may change connections when spontaneous activity still dominates. For example, infants are born with preferences for sounds present in their mother's language suggesting that before birth (when spontaneous activity plays a dominant role in shaping connections) circuitry may also be modulated by auditory input that filters into the uterus. Whether the mechanisms responsible for synaptic refinement are the same for endogenous and sensory-driven input has yet to be determined.

Mechanisms of activity-dependent competition

Physiological and structural synaptic changes

What molecular mechanisms operate so that correlated activity can drive the refinement of synaptic connections? The process is competitive in the sense that direct or indirect interactions between incoming axons for common postsynaptic targets drive the retraction of all but one (or one class of) axon and allow expansion and stabilization of the remaining axonal terminals. Ideas about how this process occurs on a cellular and molecular level in the developing CNS have drawn from primarily two sources: studies on synapse formation and elimination at the NMJ, and studies on long-term changes in synaptic strength in the hippocampus (both reviewed in Cohen-Cory, 2002). More recently, molecular mechanisms have begun to be identified in the developing visual system itself (reviewed by Taha & Stryker, 2005; Huberman, 2007). Although the details of each of these systems di er, they share a common theme: synaptic refinement depends on a mechanism in which synapses that are synchronously active with the postsynaptic cell are reinforced (hebbian synapses) whereas those synapses that are not synchronously active are eliminated (Fig. 10.2*a*).

The individual steps of synaptic refinement have been best studied at the NMJ (reviewed in Nguyen & Lichtman, 1996). As in the visual system, axonal projections – in this case from motor neurons – are initially di use, with individual muscle fibers receiving innervation from multiple motor neurons before birth. These connections are then refined through an activity-dependent process so that each muscle fiber receives input from only one axon (possibly driven initially by the spontaneous patterns of activity generated by the developing spinal cord as mentioned above). The process of synaptic refinement at the NMJ has been

monitored elegantly in vivo. Neurotransmitter receptor sites on the postsynaptic membrane (i.e., the muscle's acetylcholine receptors) first decrease, the presynaptic axon's terminals are eliminated from the muscle, and finally the nerve terminal resorbs into the main axonal trunk. Asynchronous receptor activation seems to be the signal that allows the muscle to destabilize particular synaptic sites: when focal postsynaptic blockade is produced by application of α-bungarotoxin (which blocks only the postsynaptic acetylcholine receptors, and not presynaptic transmitter release), these blocked synapses are selectively eliminated. Elimination of the blocked synapse only occurs when there is activity at the rest of the junction, suggesting that active synaptic sites are stabilized and that this activity must somehow destabilize inactive sites. Studies of neurons and myocytes in tissue culture show similar results. Stimulation of one innervating neuron can suppress transmission from the synapse of a second, inactive neuron, whereas simultaneous stimulation of both neurons results in either strengthening of transmission at both synapses or in no change. These results led to the proposal of the following scenario: when synapses are active they are somehow protected from elimination, but when they are inactive they receive a "withdrawal" signal. When neither input is active, both remain stationary because no withdrawal signals are present; when both axons are simultaneously active, they are both protected from elimination (Fig. 10.2*b*). This dependence on the balance of activity, not on activity per se, is strikingly similar to the requirement for patterned activity discussed above for the developing visual system and has shaped our hypotheses about how axons in the visual system may be pruned away during periods of refinement. How this physiological and anatomical pruning occurs at a molecular level is a current area of exciting investigation.

Molecular basis of synaptic refinement

Two key molecular steps underlying synaptic refinement have been extensively explored: first, the need to detect the coincident activity that instructs the refinement, and second, the signal that leads to the actual removal of the connection. The past decade has seen a remarkable increase in our understanding of these processes in the developing visual system.

Coincidence detection

Our understanding of the cellular and molecular correlates of coincidence detection mechanisms has been primarily shaped by many years of study on synaptic plasticity seen in the mature hippocampus (reviewed in Malenka & Bear, 2004). Long-term potentiation (LTP), the lasting increases in synaptic strength seen after the application of high-frequency electrical stimulation in the hippocampal CA1 region, is the best-studied form of synaptic strengthening. In contrast, synaptic weakening can be produced by lower frequency stimulation resulting in long-term depression (LTD). LTP is an appealing mechanism for synaptic modification during development because it requires cooperativity (a certain number of synapses must be active to induce LTP), associativity (the coincident firing of a weak synapse along with stronger synapses on a convergent input will potentiate both sets of synapses), and input specificity (only synchronously active inputs are potentiated), all of which are thought to operate at developing synapses. Additionally, LTP and LTD can occur homosynaptically (at the same synapse that is stimulated) and heterosynaptically (at other synapses on the same postsynaptic cell that is being depolarized by the active synapse), allowing for the strengthening and weakening of multiple synapses, as is necessary in the refinement of developing connections (Fig. 10.2). These attributes have led to an exploration of the roles of LTP and LTD as mechanisms underlying synaptic refinement during development.

In the developing visual system, both retinogeniculate and geniculocortical synapses demonstrate forms of synaptic enhancement similar to hippocampal synapses (Mooney *et al.*, 1993; Crair & Malenka, 1995). Developing LGN synapses can undergo long-term increases in transmission with high-frequency stimulation or pairing of presynaptic and postsynaptic activity (Mooney *et al.*, 1993). However, low-frequency activity from retinal axons does not seem to induce LTD in the developing LGN (Firth *et al.*, 2005). In the developing neocortex, LTP has been demonstrated directly at the geniculocortical synapse (Crair & Malenka, 1995). LTD has also been described in the neocortex (Dudek & Friedlander, 1996). Both LTP and LTD are easier to elicit at synapses during the period of synaptic refinement in the cortex (Crair & Malenka, 1995; Dudek & Friedlander, 1996).

One molecular requirement that is necessary for LTP and LTD is a physiological "coincidence detector," a mechanism by which coincident activity of the presynaptic and postsynaptic neurons is sensed (Fig. 10.2*b*). The glutamatergic *N*-methyl-D-aspartate

(NMDA) receptor is a molecular coincidence detector that has been studied in great detail because it can detect coincident activity (see Bear, 1995). The NMDA receptor uxes Ca^{2+} ions only when glutamate, released by the active presynaptic neuron, binds to the receptor and the postsynaptic neuron is depolarized simultaneously, relieving a voltage-dependent Mg^{2+} gating of the channel pore. The level of Ca^{2+} in ux into the postsynaptic neurons appears to determine whether LTP (high Ca^{2+}) or LTD (low Ca^{2+}) occurs. When NMDA receptors are blocked, many forms of LTP and LTD cannot be elicited.

Like the hippocampal synapse, the retinogeniculate and thalamocortical synapses use glutamate as their neurotransmitter, which has led to an investigation of which glutamate receptors are critical for synaptic refinement during visual system development. At the retinogeniculate synapse, physiological synaptic enhancement appears to depend on NMDA receptor activation, at least in some neurons (Mooney *et al.*, 1993). In the developing cortex, in vivo infusions of NMDA receptor antagonists block the physiological shifts in ODCs produced by monocular blockade (although the specificity of NMDA receptor blockade in the cortex has been called into question because these antagonists also block most activity in developing cortical neurons). The requirement for NMDA receptor-mediated plasticity is perhaps best defined for the optic tectum of lower vertebrates. In the three-eyed frog model, the eye-specific stripes in the tectum not only desegregate when all activity is blocked with TTX, as discussed earlier, they also desegregate when NMDA receptor antagonists are applied (reviewed in Cline, 1991). However, although NMDA receptor-mediated plasticity has been most thoroughly studied, there are many examples of plasticity mechanisms that do not require NMDA receptors (reviewed in Malenka & Bear, 2004). NMDA receptors in all brain regions do not undergo activity-dependent refinement so additional mechanisms of coincidence detection will likely be critical for activity-dependent synaptic refinement.

The functioning of particular mechanisms for coincidence detection, such as a reliance on glutamate receptors, may increase the susceptibility of the developing brain to particular insults. In addition, recent experiments suggest that the earliest synapses that form during the development or rearrangement of glutamatergic circuits may contain solely NMDA receptors (see Poncer, 2003). In the hippocampus, such synapses have been dubbed "silent synapses" because they are undetectable at resting membrane potentials, but the NMDA receptor-dependent excitatory postsynaptic potentials are revealed when the postsynaptic cell is depolarized. After LTP induction protocols, α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) receptors appear at these synapses so that they are no longer functionally silent. It is possible that the severe disruption caused by glutamate or glycine toxicity in the developing brain is caused not only by acute cell death, but additionally by the disruption of this first step in the refinement of synaptic connections. Exacerbating this disruption may be the role that presynaptic activity can play in the regulation of postsynaptic ion receptor subtypes, which control ion ow and neuronal excitability (Spitzer *et al.*, 1994). Changes in the properties of the glutamate receptor subunit composition during development also allow for increased Ca^{2+} ux through these receptors. Neurons with glutamatergic synapses may be prone to death by toxicity because of excessive Ca^{2+} entry after the release of large amounts of glutamate during a hypoxic–ischemic episode, through activation of either NMDA receptors or AMPA receptors (Grow & Barks, 2002). Similar damage can also follow the release of glycine (which at high levels can stimulate NMDA receptors) as seen in genetic disorders such as nonketotic hyperglycemia (Grow & Barks, 2002). The specific physiology of the developing circuitry is presumably intimately related to morphological changes that are later seen in the brain, both in normal development and after injury.

Retrograde signals: old and new candidates

Coincidence detection allows for anterograde information ow about correlated activity between the presynaptic and postsynaptic neurons (Fig. 10.2*b*). For this activity to result in a structural change in presynaptic connections during synaptic refinement, it seems there must also be a retrograde signal that allows communication back from the postsynaptic neuron to the active presynaptic terminals (Fig. 10.2*b*). Such a signal would need to be regulated by neuronal activity and cause selective stabilization and growth at synapses that are simultaneously active with the postsynaptic neuron. Much work in the past decade focused on neurotrophins as key molecular components since they promote axon growth and their levels change in response to

neural activity (reviewed in Taha & Stryker, 2005). Neurotrophins have been joined in recent years by a much less expected category of candidate molecules – immune system components – that may translate activity levels into axon refinement.

The evidence that neurotrophins, particularly brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-4, have a role in connection refinement is now quite strong (Shatz, 1997; Taha & Stryker, 2005). The receptors for BDNF and NT-4 (TrkB receptors) are expressed in the mammalian LGN and cortex during the appropriate developmental period (TrkC receptors, which bind NT-3, are also expressed). Infusion of BDNF and NT-4 (but not other neurotrophins such as nerve growth factor or NT-3) into the visual cortex during the period of ODC development has been shown to block ODCs in the region of the infusion; blockade of the TrkB receptor also blocked ODC formation. Putting NT-4, but not BDNF or NT-3, into the visual cortex can also rescue LGN neurons from the shrinkage of their cell bodies that occurs with monocular deprivation. These experiments (reviewed in Shatz, 1997) suggest that the competitive interactions that normally underlie synaptic remodeling are based on a limited supply of neurotrophins, and infusion of excess neurotrophins acted on the LGN axon terminals, removing this competition. When there is no activity, as in monocular deprivation, then the application of NT-4 can maintain the silenced LGN neurons, although it would be predicted that their terminals would still not have been able to segregate into ODC. Whether BDNF or NT-4 functions endogenously during development has yet to be determined.

The recent unexpected finding that several immune system molecules may play a role in the developing brain has opened up an entirely new direction in the search for molecules underlying refinement. The class I major histocompatibility complex (MHC) genes (Huh *et al*., 2000; Goddard *et al*., 2007), neuronal petraxins (Bjartmar *et al*., 2006) and, most recently, members of the complement cascade (Stevens *et al*., 2007) have all been found to have roles in synaptic refinement, including in the developing visual system. In the immune system, these families of molecules have very broad functions in clearance and scavenging of infected or otherwise damaged cells. Although these molecular families are also broadly expressed in the developing brain, alterations of their expression in genetically modified mice can specifically disrupt the precise pattern of connections in the developing LGN and cortex. Even in the presence of normal retinal activity, LGN segregation does not occur in mice missing these immune system molecules. In addition, electrophysiology studies combined with anatomical assessment show that hippocampal LTP is impaired, at least in mice expressing low levels of MHC, and these molecules act as retrograde signals (Goddard *et al*., 2007) (Fig. 10.2*b*). How expression of these molecules results in axonal refinement is not yet clear, but the suggestion that neural activity, or lack thereof, causes these molecules to tag specific synapses for maintenance (Huh *et al*., 2000) or clearance (Stevens *et al*., 2007) is an intriguing parallel to their roles in the immune system. Several common neurological disorders, such as autism and schizophrenia, have been linked epidemiologically to immune system activation during development. The use of immune molecules to pattern neural connections may finally provide a causal link between pre- or postnatal immune activation and very specific changes in the developing brain.

While neurotrophins and immune molecules may promote or remove synapses, respectively, neither type of molecule is likely to function on the 10- to 100-ms scale on which coincidence detection must occur. The Trk receptor is likely to remain activated for a prolonged period once neurotrophin binds to it (Shatz, 1997) and it is likely that the immune molecules also function on a longer timescale. Nitric oxide, a rapidly di usible gas that can regulate hippocampal LTP, is a more rapidly acting candidate that has been postulated to rapidly change synaptic strength during development. However, several experiments indicate that systemic blockade of nitric oxide synthesis can block refinement of only a restricted set of connections (Taha & Stryker, 2005). The hunt for rapidly acting retrograde indicators of correlated activity continues.

Many molecular messengers can modify activity-dependent development and more messengers are yet to be identified. In the future, it will be necessary to balance potential therapeutic e ects of manipulating molecular messengers against the potential risk of creating abnormal neural circuitry.

Concluding remarks

Links are now being forged between normally occurring activity-dependent plasticity and specific developmental brain disorders (reviewed in Johnston, 2004). The reorganization of hippocampal connections after

prolonged epilepsy and the activity-dependent translation of fragile X mental retardation protein at synapses are two examples linking normal and pathological mechanisms. In the hippocampus, rearrangement and sprouting of mossy fiber connections is a striking feature of temporal lobe epilepsy; current work is focusing on the similarity of the mechanisms that appear to underlie activity-dependent formation of hippocampal connections during development and the pathologic sprouting seen after seizures. The link between the expression of the fragile X mental retardation protein and its role in normal development remains unknown, but its expression appears at least to be regulated by neural activity and could play a role in the initial formation of neural circuits that might in turn be disrupted when a pathological form of the protein is produced. Information about the role of activity-dependent developmental changes in neurological disease is just starting to emerge, but understanding normal activity-dependent developmental processes at the cellular and molecular levels should provide clues to human neurological illness that may result from changes in the circuitry of the developing nervous system.

Activity-dependent development can be disrupted at many points. We have indicated a critical role for correlated activity, both spontaneous and sensory derived, in driving neuronal competition that shapes precise connectivity. Either subtle disruptions in the spatiotemporal patterns of activity or major disruptions in the overall balance of activity may result in abnormal connectivity. Such disruption of patterned neural activity may result from interference with synaptic transmission, with coincidence detection, or with retrograde signals, as might happen with prepartum use of drugs (such as nicotine, benzodiazepines, or narcotics), postnatal exposure to a variety of medications or immune system activation during development. In addition, as di erent sensory systems become active at particular developmental stages in premature infants, it is worth considering what e ect the sensory environment might have on neural activity patterns needed to drive synaptic remodeling. During critical periods, specific areas of the developing nervous system may be particularly susceptible to these types of disruption.

Disruptions of activity would a ect the fine-tuning of neural circuits, a process that requires neural activity to shape the adult patterns of connectivity, not the large-scale wiring of the brain, which generally occurs independent of neural activity. It is all too common for a child to have neurological deficits when no structural abnormalities can be identified. The current techniques in neuropathology and neuroradiology are just beginning to be able to detect the subtle, yet critical, changes that must be occurring. Consequently, the major challenge for the clinician and medical scientist is to develop techniques with high resolution that can monitor the normal or abnormal functioning of neural circuits on a fine-scale during development. Once disorders resulting from disruptions in activity-dependent development can be more accurately identified, therapies aimed at correcting the abnormal circuits (for example, by developing treatments to extend critical periods) or optimizing the use of the abnormal circuitry (as is being tried for specific language impairments; Tallal, 2004) can be further developed. Recognizing and understanding in detail the role of activity-dependent development in the formation of precise neural circuits should expand greatly the ability to identify, treat, and prevent many developmental brain disorders.

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Historic box 5 Critical periods
Hugo Lagercrantz

The question of the role of visual experience in normal development has been raised and discussed by philosophers since the time of Descartes according to the Nobel Lecture by Torsten Wiesel in 1981. Wiesel and David Hubel were awarded the Nobel Prize in physiology or medicine "for their discoveries concerning information processing in the visual system." They were intrigued how the highly specific responses of the neurons in the visual cortex emerged during postnatal development. Furthermore they were aware of the observations that children with congenital cataract could not see well in spite of the removal of the lenses and proper refraction. It had also been demonstrated that animals raised in a dark or an environment devoid of contours develop impaired visual function.

To investigate this problem further, Wiesel and Huber decided to fuse the lids with sutures. Rearing kittens in total darkness was found to be difficult. Only one eye was closed while the other eye served as control. Wiesel and Huber then found that those kittens with one eye occluded during the first three months of life became blind in the deprived eye and that the cells in the striate cortex responded only to stimulation of the normal eye. As pointed out by Wiesel (in 1981): "This design turned out to be fortunate because the effects of single eye closure on the visual cortex are more dramatic than the results obtained from animals raised with both eyes occluded or kept in dark." These experiments demonstrated that neural connections present early in life can be modified by visual experience. These initial findings were further corroborated in rhesus monkeys.

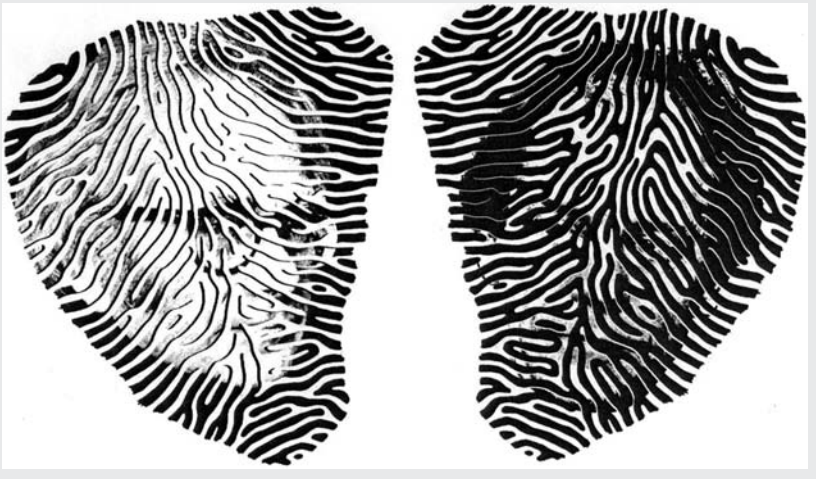

Fig. HB5 David Hubel (left) and Torsten Wiesel (right) are superimposed on the alternating patterns of eye dominance regions over the visual cortex. From Nicholas Wade.

Torsten Wiesel started his career as a doctor in Sweden, working in mental hospitals and also at a pediatric psychiatry ward. As he wanted to write a Ph.D. thesis, he became a graduate student in physiology at the Karolinska Institute. When the famous neurophysiologist Stephen Kuffler visited Stockholm to recruit a postdoctoral student, Torsten Wiesel announced his interest as the most advanced candidate was getting married. Kuffler had just published his classical study of the receptive field arrangements in the ganglion cells. Wiesel met David Hubel in the early 1960s in Kuffler's laboratory.

David Hubel grew up in Montreal, where he studied medicine at McGill University. During this time, he spent his summers at the Neurological Institute and he also spent a year in clinical neurophysiology under Herbert Jasper. This was during the period of culmination of the work of Penfield and Jasper. He then moved to Stephen Kuffler's laboratory at the Johns Hopkins University, where he started his 23-year collaboration with Torsten Wiesel, which continued at Harvard.

Walking, cycling, sensorimotor coordination, language acquisition, playing the violin or the piano are best learned during certain critical periods or windows of opportunity. Although Piaget was aware that certain ages were most important for learning various activities, Hubel and Wiesel presented the first scientific evidence showing the importance of stimulation of the infant brain during critical periods.

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Chapter 11

Fetal and neonatal development of the auditory system

S. Allen Counter

Introduction: traditional and modern biological approaches to the development of the ear

The ear is a doorway to the brain – an extension of the brain that reaches out to the periphery to collect and form structural images of sounds in the milieu. The ear informs the brain and hence the organism of the imperative sounds of development, communication, and survival. The complex hearing apparatus that forms the "ear" provides the foundation for auditory interactions with the environment and serves as the essential component of two-way communication between organisms. In mammals, the mechanical vibrations of air particles that form the physical dimension of sound are collected by the external ear, and amplified and channeled through a narrow canal within the skull to reach the sensitive eardrum or tympanic membrane. The external auditory canal and the tympanic membrane form the first section of the superbly designed mammalian ear, which is an intricate sensory system composed of a series of complicated subsystems, linked in series, to perform vibro-mechanical conduction, hydrostatic pressure matching, and mechanoelectrical transduction (Fig. 11.1). Each of these unique subsystems must be intact for sound to optimally reach the eighth cranial nerve, higher auditory tracts, nuclei and auditory cortex of the brain to initiate sound perception. Auditory stimuli that are processed by the contiguous external, middle, and internal (cochlear) components of the ear system are conveyed as neuro-electrical signals to the brain by the eighth cranial nerve. Sound frequency and feature analysis through tonotopic cellular organization take place from the cochlea and first-order neurons in the eighth nerve to higher auditory centers, including the cochlear, inferior colliculus, and medial geniculate nuclei, and ultimately the auditory cortex.

During embryogenesis, each segment of the ear (from the most peripheral structures to the auditory cortex) is formed from specific cells, which follow predetermined genetic instructions, but different embryonic pathways. When these instructions are interrupted or go awry, a number of anomalies and diseases may ensue, resulting in abnormal development in one or more of the three components of the ear and central nuclei, and ultimately causing hearing impairment. Hearing loss or deafness in the neonate, infants and older children, and adults is classified as conductive (if confined to impairment of the outer and middle ear components), sensorineural (if caused by abnormalities of the cochlear or inner ear), or retrocochlear (involving anomalies of the eighth cranial nerve or nuclei and tracts of the brainstem).

Approximately 4% of the world's population under 45 years of age has a hearing impairment that is congenital or of early onset. One in 1000 children is born with serious hearing loss or deafness that is believed to be of genetic origin, with about 70% being nonsyndromic, i.e., not associated with other clinical disease patterns (Petersen & Willems, 2006). Some congenital hearing losses are conductive in nature, involving the blockage of the air-conducting external and/or middle ear components, whereas others are of sensory-neural (sensorineural) origin, involving the inner ear structures and retrocochlear neural elements. Autosomal recessive hearing impairment from defects in the inner ear is the most common form of genetic, prelingual deafness in children. Some genes responsible for autosomal dominant, fully penetrant, nonsyndromic inner ear hearing loss (about 15%) have been localized to specific chromosomes, and their mutations identified (Lynch *et al.*, 1997; Hardisty *et al.*, 1998). Experimental manipulation of these identified genes has

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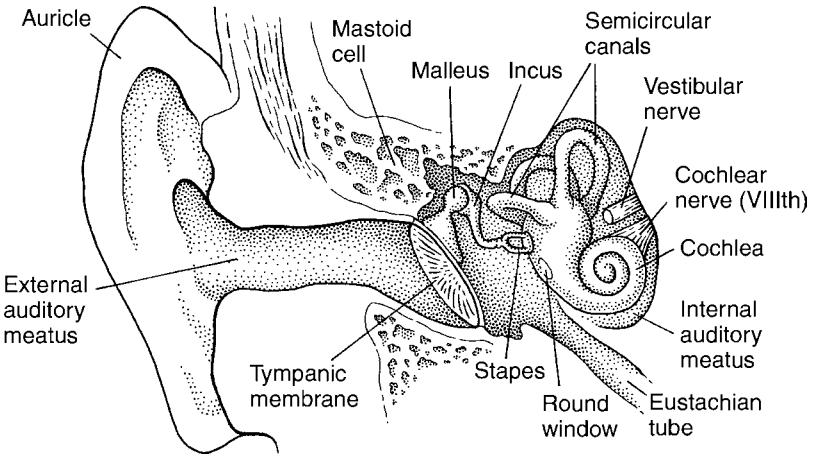


Fig. 11.1 Drawing of the human ear illustrating the external, middle, and internal ear components. The auricle or pinna, external auditory meatus, middle ear cavity (including ossicles), the inner ear (including the cochlea and semicircular canals), and the vestibular and auditory branches of the eighth cranial nerve are shown.

revealed specific effects on the morphogenesis of the ear, which have been beneficial to our understanding of the mechanisms of development and function of the entire auditory system.

The traditional approach to academic discussions of the embryogenesis of the nervous system and the organogenesis of the vertebrate ear has involved mainly a description of the anatomical formation and cellular activities in the neural precursors, neural tube, neural crest, and ectodermal placodes. Neural induction is the process of causing some ectodermal cells to become neural. Neurulation, which is the process of neural folding to form the neural tube, involves the specification of ectodermal cells into neural precursor cells. These processes are now known to be mediated by specific gene expressions. The differentiation of the ectodermal neural plate into the neural tube has been shown to follow a programmed pattern of specific cell fates, including the specification of dorsal versus ventral. Earlier descriptions of the morphogenesis and histogenesis of the ear have focused on the structural integrity and detailed embryonic changes in anatomical structure of the developing ear (Deol, 1966; Anson & Davies, 1980; Moore, 1988; Gulya, 1990; Sadler, 1990). Recent findings, however, in molecular biology and molecular genetics have added a new dimension to the discourse on fetal development and to our understanding of the molecular mechanisms underlying embryogenesis of the human ear (Fekete & Campero, 2007).

Much of our present understanding of the neurogenetic factors involved in the embryogenesis of the human ear derives from studies of morphogenesis in invertebrates such as *Drosophila* (Lewis, 1978) and some lower vertebrates (for reviews, see Fritzsch, 1996a). The comparatively "simpler" ears of certain amphibians, and avian species, for example, have served as useful models for a better understanding of the development and physiological mechanisms of the mammalian ear (Counter & Tsao, 1986; Whitehead, 1986; Borg & Counter, 1989; Fekete, 1996; Wu & Oh, 1996; Fekete & Campero, 2007).

The embryonic and postnatal formation of the mammalian ear has been studied extensively in the mouse model (Deol, 1966, 1980; Sher, 1971; Fritzsch *et al.*, 2005a). Recent findings on inner ear development in lower mammals have led to the identification of hundreds of deafness-associated genes in humans (Van Camp *et al.*, 2006.). In mice, several molecules have been identified that are involved with cell fate and morphogenesis of the ear (Léon *et al.*, 2004). These findings have been based mainly on studies of gene mutations in knockout mice and the cloning of genes for deafness (Fekete, 1999; Karis *et al.*, 2001; Xiang *et al.*, 2003). While some of the experimental gene deletions and mutations alter the formation of the outer and middle ear subsystems, others may inhibit neurogenesis and cellular formation of receptors in the cochlea (Bermingham *et al.*, 1999; Xiang *et al.*, 2003; Fritzsch *et al.*, 2005b). Hereditary deafness has become a major focus of research in the embryogenesis of the auditory system (Brown & Steel, 1994). More recent advances in molecular biology have revealed the contributions of a number of inductive factors and specific genes to the patterning of the cellular aggregates at each stage of the embryogenesis of the ear. Some 60 genes have been identified that are associated with ear abnormalities and deafness. These anatomical and physiological anomalies are seen in the outer, middle, and inner ear components at various

stages of embryogenesis (Deol, 1980; McPhee & Van De Water, 1988; Ruben *et al.*, 1991; Ekker *et al.*, 1992; Keynes & Krumlauf, 1994; Gallagher *et al.*, 1996; Fritzsch, 1996a, b; Fekete, 1996, 1999; Whitfield *et al.*, 1996; Holme & Steel, 1999; Karis *et al.*, 2001; Wright & Mansour, 2003).

The embryonic and fetal development of the human auditory system follows a precise extended timetable over the period of gestation in which the vital skeletal, muscular, and neural structures are formed and mature to functional status. From the formation of the otic placode in the third week of gestation and the formation of the otocyst to the growth of the mesenchymal and endodermal tissues that form the outer and middle ear structures, the embryogenesis of the ear is guided predictably by intrinsic genetic inductive cues (Figs. 11.2 and 11.3). Each intricate subsystem must develop properly to form a seamless mechanoelectrical

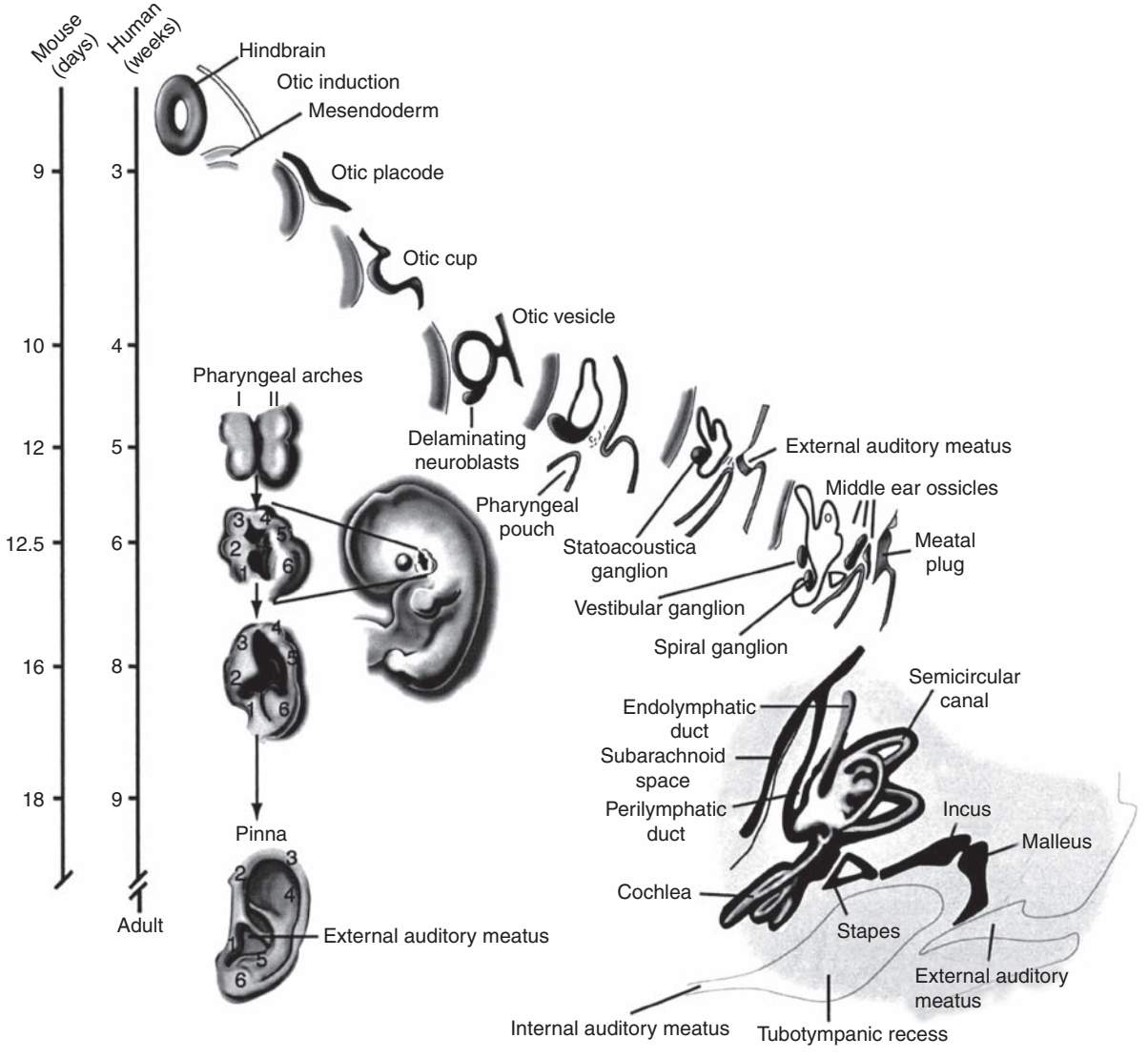


Fig. 11.2 Timetable of ear development. Inner ear induction begins just prior to the third week of pregnancy in humans. The major morphogenetic changes in the ectodermal components of the inner ear occur over the following five to six weeks. Interactions with and remodeling of the surrounding periotic mesenchyme continues for several months. Meanwhile, beginning at about the fifth week of gestation, the development of the middle and outer ears ensues. These two parts of the ear interact to form the tympanic membrane between the external auditory meatus and the tubotympanic recess of the middle ear cavity. The external auditory meatus originates at the cleft between the first and second pharyngeal arches whose hillocks will morph into the pinna. The approximate ages at which these major events are observed in the mouse is also indicated on the far left. (From Fekete, 2009; modified with permission.)

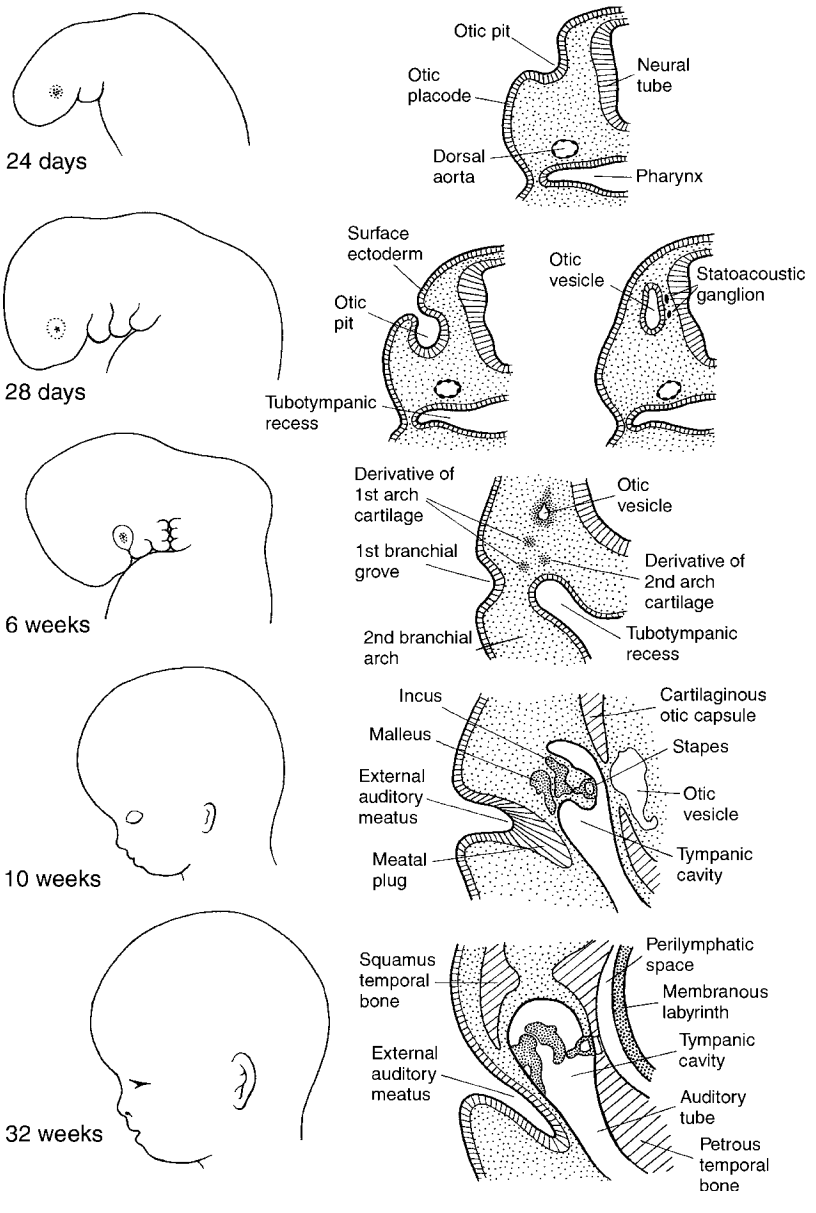


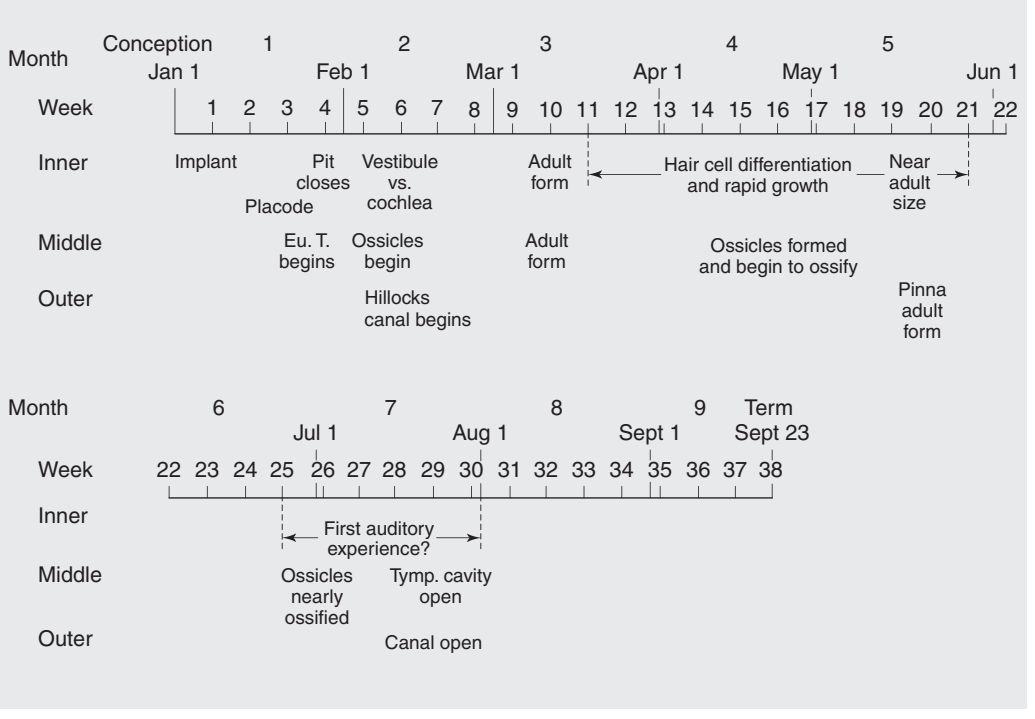
Fig. 11.3 Classical drawing of the sequence of developmental changes in the fetus from day 24 involved in the formation of the outer and middle ear, showing the initial rhombencephalon invagination of the otic placode and otic pit and the appearance of the external auditory meatus and middle ear ossicles.

sense organ in order for the auditory system to function normally. A defect in any component of the outer, middle, or inner ear may hinder the signal transmission capacity of the hearing organ and lead to deafness. Embryonic development of the ear may be disrupted by a variety of factors such as prenatal bacterial or viral infections, including bacterial meningitis and cytomegalovirus (CMV) infection. For example, congenital CMV infection is believed to be a frequent cause of severe sensorineural (inner ear or cochlear) hearing loss in neonates (Pappas, 1983). Also, human fetuses that are exposed to rubella in the first trimester of pregnancy may develop a remarkable bilateral sensorineural hearing loss, or in some cases profound deafness.

The inner ear

Since the end organ of hearing, the cochlea, is the first of the subsystems to develop during embryogenesis of the ear, the development of the inner ear will be reviewed first. Table 11.1 (modified from Peck [1995]

Table 11.1 Timetable of the embryological formation of the human ear from conception to term[a]



[a] Modified from Peck (1995) with permission.

with permission) shows a timetable for the development of each subsystem of the human ear. Congenital malformations of the inner ear may result in moderate to severe sensorineural hearing loss or complete deafness (Jackler *et al.*, 1987).

The embryological development of the mammalian cochlea, like that of other sensory systems, proceeds in close association with the development of the central nervous system. The intricate inner ear consists of the organ of Corti, afferent and efferent neuronal endings of the auditory nerve, and the vestibular components – the otolith organs (the utricle and saccule), the endolymphatic duct and sac, and the three semicircular canals. Each of these structures is contained within a membranous labyrinth, which is housed in a surrounding bony labyrinth. The mechanoelectrical receptors of both the cochlea and vestibular organs are innervated by afferent fibers (otic placode) of the cochlear and vestibular ganglion cells. The receptors receive efferent neurons (from the brainstem basal plate) and autonomic nerve elements from the superior cervical ganglia (from the neural plate) (see Fritzsch *et al.*, 1997). The vestibular portion of the inner ear is an integral but separate conglomerate of sensory organs that serve balance, spatial position, and acceleration, and as such will not be covered in this section on the inner ear.

The mammalian cochlea, which is embedded in the temporal bone, is a unit of coiled fluid-filled chambers or scalae (steps). The three chambers are the scala vestibuli, the scala media, and the scala tympani (Fig. 11.4*a*). The fluid of the scala media, the endolymph, is potassium-rich and similar to cerebrospinal fluid in its protein composition, while the perilymph of the scala vestibuli and scala tympani has a substantially higher concentration of sodium and protein. The scala media or cochlear duct contains the organ of Corti, which has a single row of differentiated epithelial cells called the inner hair cells (around 3500), and three rows of approximately 20 000 outer hair cells (Fig. 11.4*b*, *c*). The inner and outer hair cells interface with supportive cells and pillar cells which are separated by a tunnel (tunnel of Corti). On their apical surface, the hair cells contain contractile proteins, including an actin cuticular plate and about 100 stereocilia, which are graduated in their length, and which project to the overlying tectorial membrane.

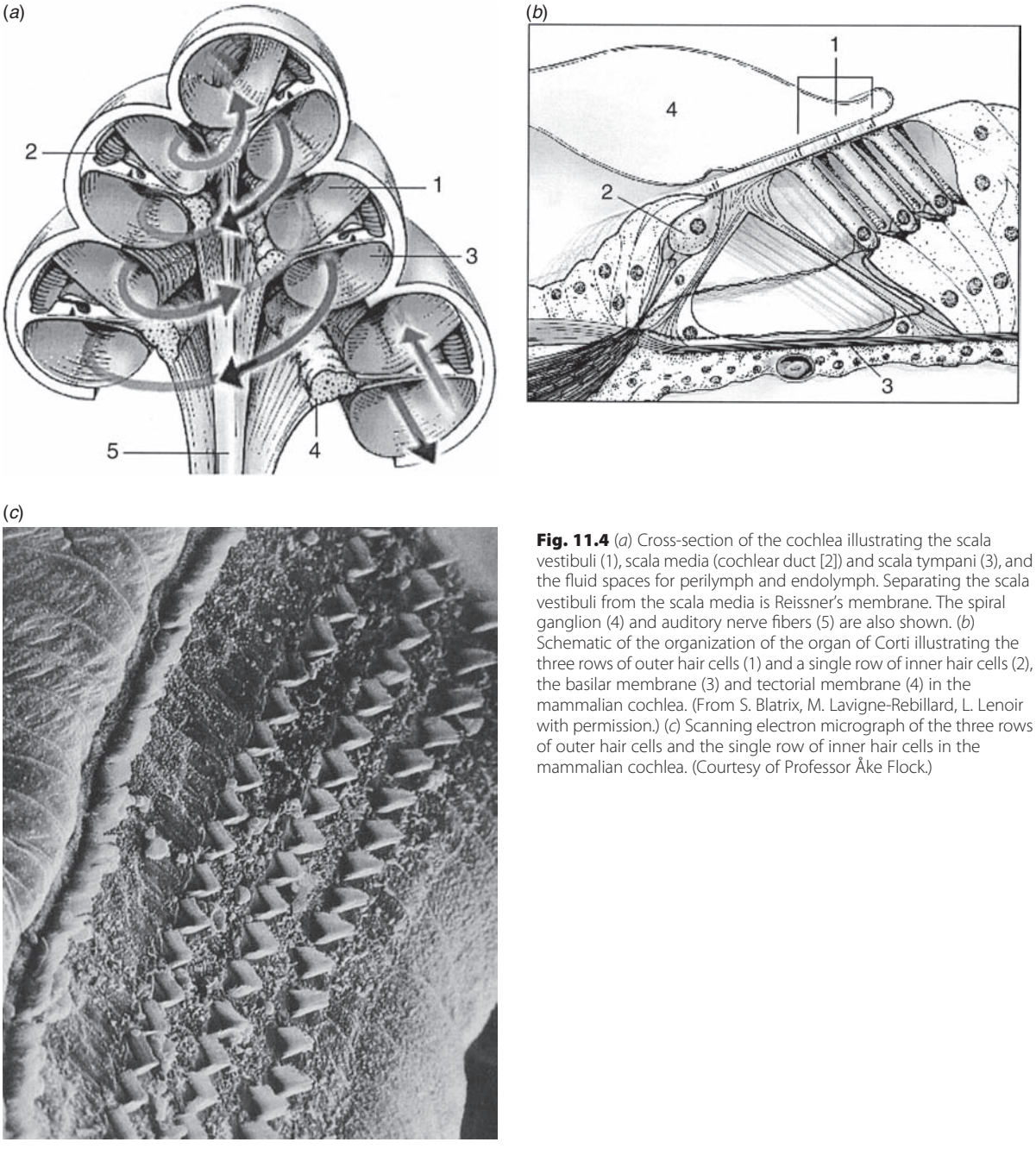


Fig. 11.4 (*a*) Cross-section of the cochlea illustrating the scala vestibuli (1), scala media (cochlear duct [2]) and scala tympani (3), and the fluid spaces for perilymph and endolymph. Separating the scala vestibuli from the scala media is Reissner's membrane. The spiral ganglion (4) and auditory nerve fibers (5) are also shown. (*b*) Schematic of the organization of the organ of Corti illustrating the three rows of outer hair cells (1) and a single row of inner hair cells (2), the basilar membrane (3) and tectorial membrane (4) in the mammalian cochlea. (From S. Blatrix, M. Lavigne-Rebillard, L. Lenoir with permission.) (*c*) Scanning electron micrograph of the three rows of outer hair cells and the single row of inner hair cells in the mammalian cochlea. (Courtesy of Professor Åke Flock.)

The stereocilia are composed of the active contractile proteins actin and myosin (Flock & Cheung, 1977; Flock, 1980). The stretching of the links of the tips of the stereocilia in relation to the tectorial membrane during sound-induced mechanical vibrations in the perilymphatic and endolymphatic fluids causes an opening in stereocilia transduction channels, an influx of endolymphatic K^+ at the hair cell, and, ultimately, depolarization and release of neurotransmitter substance for synaptic communication with neurons at their base. The myosin, which is involved with hair cell adaptation and passive channel opening, works in conjunction with actin through adenosine triphosphate (ATP) hydrolysis to provide a power force, and

to readjust the tip links. The hair cells, their stereocilia and tectorial membrane contacts convert through their motile actions the fluid disturbance caused by external sound waves to electrical activity in the contiguous synapses at their base (Hudspeth, 1989). The electromechanical features of the outer hair cells, including their motility, are believed to underlie the frequency selectivity of the hair cells. The outer hair cells are an important physiological component of the cochlear amplifier, which is involved with the fine tuning of hearing.

The inner and outer hair cells of the cochlear duct are innervated at their base by afferent and efferent fibers from the eighth cranial nerve. Type I afferent auditory fibers (90%) are myelinated and innervate the inner hair cells. Type II fibers are unmyelinated and communicate with the outer hair cells. The efferent or centrifugal neurons, which inhibit mechano-electrical transduction at the receptor level, originate in the brainstem superior olivary complex and innervate the inner and outer hair cells throughout the cochlea. The lateral olivocochlear bundle innervates the inner hair cells, and the medial olivocochlear bundle serves the outer hair cells. The hair cells convert sound-induced mechanical disturbances in the fluid and membranes of the scala media into neural impulses in the cochlear nerve. The inner and outer hair cells permit a broad range of sensitivity to sound, with the latter being more sensitive to a wide spectrum of sound intensities and frequencies. About 25 000 bipolar neurons in the cochlear nerve transmit the digital information of all sounds, including language, from the auditory periphery to the brain.

The embryogenesis of the inner ear initiates from interaction of hindbrain inductive fields between the otocyst and the neural tube. The embryonic development of the mammalian inner ear begins at 25 days with a thickening of the surface ectoderm on the side of the head of the embryo called the otic placode, which emerges bilaterally at rhombomeres 5 and 6 on the caudal hindbrain (myelencephalon). The embryonic rhombencephalon and the chordamesoderm exert inductive influences on otic placode specification and invagination with fibroblast growth factors playing a critical role (Peck, 1994; Fekete, 1999, 2009). The otic placode invaginates to form the otic pit which when fused at the mouth becomes the otocyst or hollow otic vesicle. The surface otic placode is formed at approximately 28 days when it begins to invaginate to form the otic pit, which is still open to the surface (Figs. 11.2 and 11.3). When the surface of the otic pit is fully closed, the otic vesicle (otocyst) is formed. The otic vesicle gives rise to the cochlea, its epithelial lining and the membranous labyrinth. The communication of inductive signals between the otocyst and the adjacent neural tube is necessary for the development of the membranous labyrinth. After several days, the otocyst divides into dorsal and ventral segments that will eventually become the six sensory organs of the inner ear: the vestibular apparatus (three semicircular canals, saccule, and utricle) and auditory (cochlear) parts, respectively (Anniko & Wikström, 1984; Sadler, 1985; McPhee & Van de Water, 1988; Moore *et al.*, 1988; Van de Water, 1988; Van de Water & Repressa, 1991; Peck, 1994; O'Rahilly & Müller, 1996; Fekete, 1996, 1999, 2008).

Development of the utricle, endolymphatic duct, saccule, cochlea, and semicircular canals is associated with a localized distribution of apoptosis (Nishikori *et al.*, 1999). At approximately 34 days, morphogenesis begins: the endolymphatic appendage appears dorsally and the cochlear duct elongates ventrally (Fig. 11.5). The organ of Corti complex develops in the basal sections first, followed by the mid and apical regions. Afferent and efferent synaptogenesis begins during the late embryonic stages, and may not be complete until the postnatal period.

Development of the otic capsule begins with mesenchymal cells, and is induced by actions of the otic vesicle. The mesenchyme is remodeled by cavitation of the otic capsule to form the openings of the developing bony labyrinth. The cavitation results in the formation of the scala vestibuli and the scala tympani. The capsule is transformed from its cartilaginous state to an ossified form around the 16th week of fetal development, from the base of the cochlea to the distal portions to the semicircular canals. The human inner ear reaches adult form at about 10 weeks of fetal development, and reaches adult size at around 20 weeks. During the embryological period of 9–20 weeks, the receptor cells undergo differentiation and grow to mature sizes. By the 26th week, the human inner ear is fully developed and believed to be functional (Kenna, 1990; Peck, 1995; Phippard *et al.*, 1998).

The outer ear

The outer (external) ear consists of the auricle or pinna, which collects airborne sound from the environment, and the external auditory canal which channels the sound waves toward the middle ear, and is bounded by the tympanic membrane or eardrum,

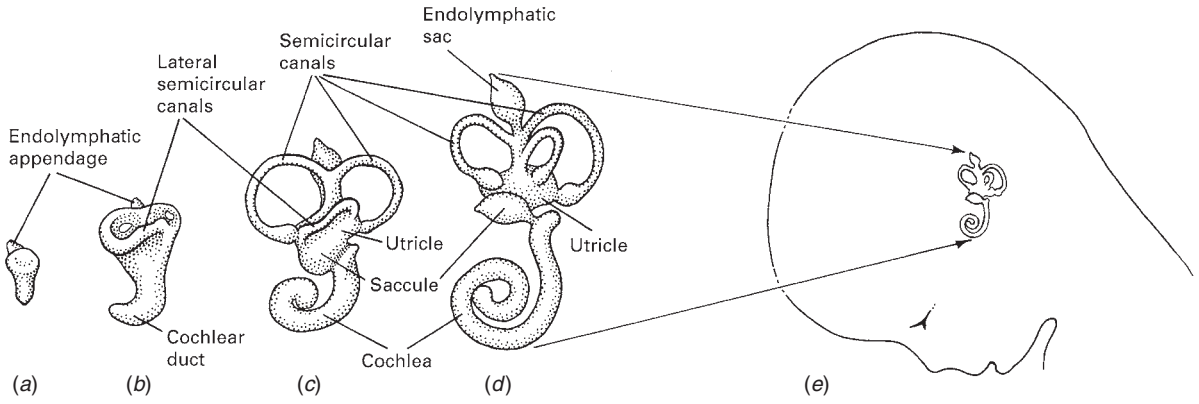


Fig. 11.5 (*a*) Schematic summary of the development of the fetal cochlea and semicircular canals of the inner ear. (*a*) The otic vessel with an emerging endolymphatic appendage. (*b*) The lateral semicircular canals and cochlear duct appear. (*c*) The spiralling of the cochlear duct begins and the three semicircular canals and endolymphatic sac are clearly visible. (*d*) The developed embryonic inner ear shows the fully spiral cochlear duct, endolymphatic sac, utricle, saccule, and the anterior, posterior and lateral semicircular ducts (canals). (Modified from O'Rahilly & Müller, 1996.)

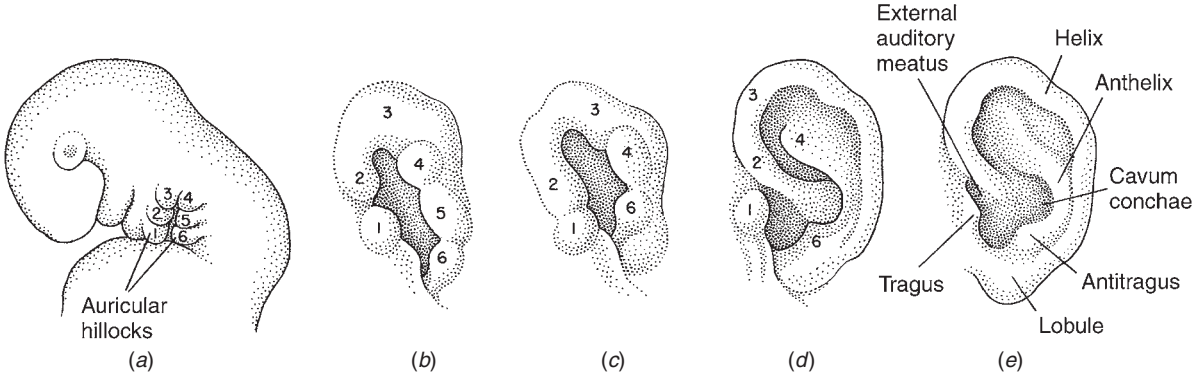


Fig. 11.6 Illustration of the progressive formation of the human auricle from the embryonic auricular hillocks (left side) to the fully developed newborn pinna (right side). The auricle or pinna collects airborne sound and focuses the collected sound waves in the narrow external auditory meatus. Each numbered hillock represents accumulations of mesenchymal tissue.

which converts the airborne vibrations to sympathetic mechanical vibrations (see Fig. 11.1). The most visible part of the external ear is the auricle which first appears in the embryo as auricular hillocks (enlargements) around the pharyngeal (or branchial) arches 1 and 2. The mesenchymal auricular swellings appear at about five weeks in embryonic development and increase in size and number to six at about six weeks (Fig. 11.6). The auricle derives from neural crest cells and mesenchyme, the embryonic connective tissue covered by cuboidal epithelium, in the first and second pharyngeal arches. The mesoderm in the first and second pharyngeal arches contributes the mesenchymal tissue in the hillocks.

The first three hillocks are the mandibular, and are believed to develop into the tragus and the crus of the helix. Auricular hillocks 4–6, the hyoid form the helix and antitragus. Over the course of the embryonic period, as the mandible develops along with other craniofacial features, the auricles migrate from a ventrolateral position at the neck region to a dorsolateral position at the side of the head and the level of the eye, with the lobule being the last part to develop (Anson & Davies, 1980; McPhee & Van De Water, 1988; Moore, 1988; O'Rahilly & Müller, 1996).

At approximately eight weeks, the external auditory meatus develops from the dorsal aspect of the pharyngeal clefts, between the auricular hillocks. The ectodermal cells in the inferior aspect of the first pharyngeal groove multiply and invaginate to form an epithelial meatal plug (Fig. 11.3). The fetal auricle and external auditory canal reach adult form at about

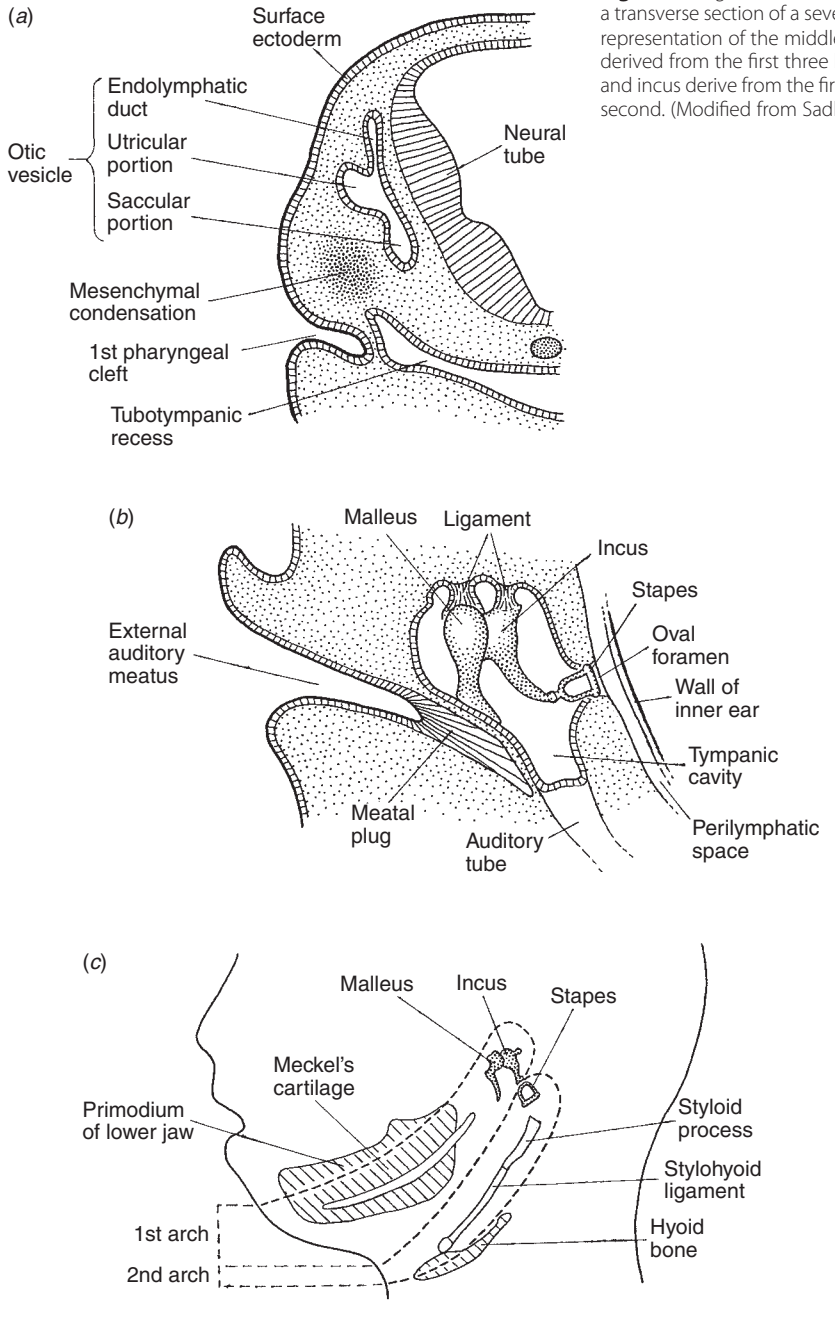


Fig. 11.7 Progressive formation of the fetal middle ear showing (*a, b*) a transverse section of a seven-week-old embryo, and (*c*) a schematic representation of the middle ear and mandibulofacial structures derived from the first three branchial arches. The auditory malleus and incus derive from the first branchial arch and the stapes from the second. (Modified from Sadler, 1990.)

20 weeks, but continue to grow in size relative to the head into the puberty years (McPhee & Van De Water, 1988), with continued size modifications through life. The tympanic membrane forms at the point of contact between the ectodermal meatal plug and the endodermal tubotympanic recess, and is constructed in three layers: an outer ectodermal layer that is continuous with the tissue of the external auditory meatus, the intermediate layer of mesoderm, and the inner ectodermal layer that is continuous with the mucous membrane of the middle ear cavity (Anson & Davies, 1980).

Congenital defects of the human external ear include atresia of the external auditory meatus due to arrested development of the meatal plug or blockage

by bone (see Fig. 11.3). Atresia of the auditory canal causes a conductive hearing loss that is amenable to surgical correction. Malformation of the auricle is also seen in disruptions of the first branchial arch (first branchial arch syndrome). For example, Treacher Collins syndrome is associated with congenital bilateral malformation of the auricles and external auditory meatus, mandibular and zygomatic hypoplasia, bilateral microtia, and associated conductive hearing loss (Bergstrom, 1980). Common anomalies of the outer ear include microtia (abnormally small auricle) and macrotia (abnormally large auricle), both of which are often associated with syndromes.

It is now known that some forms of homeotic (*Hox*) genes guide the molecular differentiation of embryonic cells to form distinct auditory structures. In mice, for example, two defective copies of the *Hoxa1* gene result in distortions in the embryonic development of the auricle and external auditory meatus. The congenital malformations seen in homozygous mutant mice probably result from a disruption of the critical inductive interactions between the mesenchyme of pharyngeal arches 1 and 2 and interactions between the arches, ear, and the hindbrain (Chisaka *et al.*, 1992). Moraes *et al.* (2005) and Arnold *et al.* (2006) have reported that Tbx1 from the T-box-containing transcription factors group is associated with outer and middle ear development. In humans with DiGeorge syndrome, and in Tbx1-null mice, the skeletal components of the outer and middle ear components are malformed. Arnold *et al.* (2006) reported that the first pharyngeal pouch (Fig. 11.3) failed to develop in *Tbx1*-knockout mice.

The middle ear

The middle ear is an air-filled cavity that serves as a hydrostatic pressure matching system and a mechanical sound transmission unit. The middle ear of mammals develops into a mucosal-lined cavity that is defined by the tympanum, which forms the boundary with the external ear, the medial bony wall, and the fenestra (windows) of the cochlea. The middle ear arises near a portion of the developing skull that contains pneumatic (air-filled) mastoid cells that increase with growth of the petromastoid, stylus, squamous and tympanic components of the temporal bone. The middle ear subsystem consists of the auditory ossicles (malleus, incus, and stapes), the ossicular muscles (tensor tympani and stapedius), the pharyngotympanic (eustachian) tube and the middle ear cavity. In the third and fourth weeks of development in the human embryo, the first pharyngeal pouch forms, giving rise to the mucosal layer of the tympanum, the tympanic cavity, and the eustachian tube (Figs. 11.2 and 11.7). The mucosal layer of the tympanum, the eustachian tube, and the tympanic cavity originates from the endoderm, while the ossicles develop from the cephalic mesenchyme. The mesenchyme makes up a large proportion of the embryonic human temporal bone and is reorganized as the cavity expands (Piza *et al.*, 1998). The tympanic cavity is lined with epithelial cells that are derived from the endoderm. The auditory ossicles, which derive from the first and second branchial (pharyngeal) arches, appear in the sixth week of human embryonic development and become fully formed in the area of the tubotympanic recess and external auditory meatus in the eighth week. After emerging from mesenchymal tissue, ossification of the auditory ossicles continues throughout fetal development, reaching full adult size in five months.

The tiny middle ear muscles which are attached to the ossicles and the middle ear cavity wall are also formed from mesenchymal tissue, and develop from the first and second branchial arches, respectively, by the 16th week (McPhee & Van De Water, 1988). The tensor tympani muscle, which inserts onto the manubrium of the malleus at the tympanic membrane, is innervated by a branch of the trigeminal (fifth cranial) nerve. The stapedius muscle inserts onto the neck of the stapes and effectively abducts the stirrup-shaped ossicle from the oval window. The stapedius is innervated by the chorda tympani branch of the facial (seventh cranial) nerve. The smallest of the skeletal muscles, the stapedius, and the tensor tympani contract reflexively in response to loud sounds and during vocalization such as an infant's cry to attenuate and filter the sound reaching the cochlea (Borg & Counter, 1989).

The eustachian tube communicates with the nasopharyngeal cavity and adjusts middle ear air pressure relative to ambient pressure. This important pressure matching system maintains air equilibration and in so doing protects the ear. For example, this automatic middle ear pressure adjustment action or the lack thereof is probably the reason for infant crying during sudden ambient air pressure changes in the landing phase of an aeroplane flight.

The middle ear is subject to a number of developmental abnormalities, including malformation of the ossicles and tympanic membrane. Perhaps the most

common childhood middle ear disorder is otitis media, which may cause conductive hearing loss, discomfort, and in chronic cases permanent damage to middle ear structures as well as the inner ear. A second common middle ear disorder is otosclerosis, an autosomal dominant disorder that results in the production of excess osseous tissue in the tympanic cavity and fixation of the footplate of the stapes in the oval window. As with otitis media, otosclerosis causes a conductive hearing loss that is amenable to medical intervention. Congenital middle ear disorders are associated with other conditions and syndromes, such as Apert acrocephalosyndactyly, Goldenhar syndrome, and Pierre Robin syndrome (Bergstrom, 1980). Studies of the development of the middle ear have revealed skeletal developmental anomalies in the bones and branchial arch of *Tbx1*-null mice (Moraes *et al.*, 2005; Arnold *et al.*, 2006).

Gene expression and neurotrophic factors in the embryonic development of the ear

During neurulation, the hindbrain or rhombencephalon exhibits a series of transient bulges called rhombomeres, which are critical for the subsequent development along different embryonic pathways of the inner, middle, and external parts of the vertebrate ear, as well as the cranial nerve ganglia (McPhee & Van De Water, 1988; Moore, 1988; Sadler, 1990; Ruben *et al.*, 1991; O'Rahilly & Müller, 1996). The inner ear, which develops first from the somatic ectoderm, can be observed adjacent to the developing rhombomere. The molecular mechanisms involved in the formation of the bony labyrinth are not completely understood. However, the *Brn4* gene has been strongly implicated in the formation of the bony labyrinth of the mammalian ear. Expression of this gene has been observed in the ventral portion of the otic capsule in the early embryonic stages (Phippard *et al.*, 1998). The middle ear and external ear components develop later. Recent evidence suggests that inductive fields or signals in the ectoderm and mesoderm activate the formation of the otic disk or placode (Vendrell *et al.*, 2000). These genetic inductive factors affect simultaneously the rhombencephalon, neural folds, and otic disk. Interruptions in rhombomere development may cause congenital defects in the ear (Chisaka *et al.*, 1992) that implicate specific gene transcription patterns in the embryological formation of the auditory outer, middle, and inner ear components (Pirvola *et al.*, 1992, 2000; Carpenter *et al.*, 1993; Repressa *et al.*, 2000).

Earlier studies of the genes from the *Drosophila* homeotic complex suggested that the homologous vertebrate homeotic *Hox* family of genes may specify segment identity in the anteroposterior axis of the mammalian embryo (Lewis, 1978; Redline *et al.*, 1992). The *Hox* cluster of genes consists of 38 members, arranged in four linkage groups (*HOX* A–D) and on four distinct chromosomes in the genome (Chisaka *et al.*, 1992). The genes can be experimentally manipulated or targeted for disruption by exogenous chemicals to study the mechanisms of expression. Using the mouse as a model for the human auditory system, Chisaka *et al.* (1992), for example, demonstrated that targeted disruption of the homeobox gene *Hox-1.6* (*Hoxa1*) resulted in severe defects in the embryonic development of the outer, middle, and inner ear, as well as the cranial nerves. Other genes such as *fgf-3* (or *int-2*), a member of the fibroblast growth factor (*FGF*) gene family, the *Pax2*, *Pax3*, and the *Otx* genes have been found to be involved in the formation of the inner ear (Torres & Giraldez, 1998; Burton *et al.*, 2004; Hatch *et al.*, 2007; Zelarayan *et al.*, 2007).

Recent findings indicate that there are several groups of genes expressed during embryogenesis of the otic vesicle: genes encoding for transcription factors, such as dlx-3; dlx-4, GH6, SOHo-1, otx1, msx-D, Nkx-5.1, PAX-2 (Burton *et al.*, 2004); genes encoding secreted factors such as FGF-3, wnt-3, Xwnt-4, Bmp-4; genes encoding receptor tyrosine kinases: ret, sek-1; and *Delta*, a gene encoding membrane proteins of the Notch signaling system (Fritzsch *et al.*, 1995, 1997; Fekete, 1996). Inductive signals expressed in the developing hindbrain at the level of rhombomeres 5 and 6, particularly int-2 (FGF-3), are required for the formation of the endolymphatic duct and sac (McKay *et al.*, 1996). Lynch and colleagues (1997) identified *DFNA1*, a human homolog of the *diaphanous* gene in *Drosophila* to be responsible for causing autosomal dominant, nonsyndromic inner ear membranous defects, and severe hearing loss. An even more distinct effect is seen in the mouse atonal homolog (*math*) 1 gene, which has been reported to be responsible for the specification of the cochlear and vestibular hair cells (Bermingham *et al.*, 1999). Two critical molecules have been identified in the induction of the otic placode. These signaling molecules – FGF-19, produced in the mesoderm, and Wnt-8c, from the neural plate – bring about the induction of the otic placode and inner ear organogenesis (Ladher *et al.*, 2000). Wright and

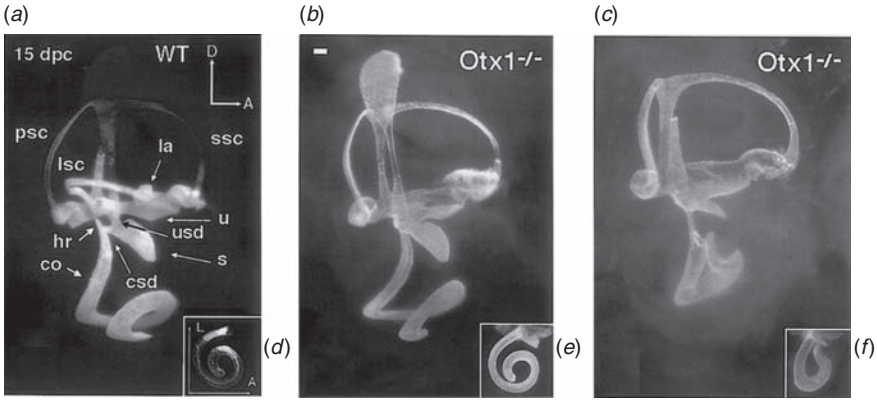


Fig. 11.8 (See color plate section.) Paint-filled inner ears of wild-type (wt) and *Pax2* mutants at 15.5 dpc. (*a*), (*b*), and (*c*) are lateral views and (*d*), (*e*), and (*f*) are anterior views of wt or mutant inner ears. (*a*, *d*) A wt inner ear showing a fairly mature morphology. (*b*, *e*) A *Pax2* homozygous inner ear with a less severe phenotype than the one in (*c*) and (*f*). The cochlea is shortened, and the common crus and the endolymphatic duct (ed) are fused. The semicircular canals are slightly thinner than wt. (*c*, *f*) A more severe form of a *Pax2* mutant inner ear with semicircular canals displaced laterally due to exencephaly. The diameter of the semicircular canals is larger than wt, and the cochlea is shortened. However, the fusion between the common crus and the ed is at a more ventral location than the specimen shown in (*e*). lsc, lateral semicircular canal; psc, posterior semicircular canal; s, saccule; u, utricle. Orientation on arrows: A, anterior; D, dorsal; L, lateral. (From Burton *et al.*, 2004 with permission.)

colleagues (2003) have demonstrated the expression of mouse *Fgf* genes and FGF receptor genes in tissues involved in inner development, with roles in induction and maintenance.

A number of neurotrophins and their receptors are critical for the normal development of cochlear and vestibular neurons (Ernfors *et al.*, 1995; Fritzsch *et al.*, 1997; Phippard *et al.*, 1998). Among the known molecules from the neurotrophin gene family that directly or indirectly modulate the afferent and efferent nerve fibers to the ear are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) (mice lacking NT-3 exhibit a reduction in cochlear neurons), and NT-4/5. These molecules bind selectively with receptor tyrosine kinases in the inner ear. NGF binds with the receptor TrkA, BDNF and NT-4/5 with TrkB, and NT-3 with TrkC (Von Bartheld *et al.*, 1991; Pirvola *et al.*, 1992, 2000; Schecterson & Bothwell, 1994; Fritzsch *et al.*, 1997). TrkB and TrkC receptors are expressed in early embryonic development of the cochlear and vestibular ganglia (Pirvola *et al.*, 1992). The BDNF and NT-3 molecules and their receptors TrkB and TrkC are essential for survival of inner ear ganglion cells and ultimately the normal innervation of the inner ear (Fritzsch *et al.*, 1995, 2004).

Other findings on the molecular mechanisms involved with normal formation of the otic capsule have shown that the human brain-4 ortholog (*Brn4*; *POU3F4*) gene is expressed in the otic capsule of mice in early otic embryogenesis. The *Brn4* gene expression is believed to be regulated as the mesenchymal condensation forms the otic capsule (Phippard *et al.*, 1998). Mutations in human *BRN4* result in cochlear and middle ear hearing loss, defects in the bony labyrinth, partial hypoplasia of the cochlea, stapes fixation, dilated internal auditory meatus, and blocked cochlear duct. The occurrence of this dysplastic phenotype in the cochlea (and internal auditory meatus) of individuals with mutations in the human ortholog *POU3F4* (around 27–47 days of gestation) appears to coincide with the timing of *Brn4* gene expression in the developing otic capsule of the mouse (de Kok *et al.*, 1995).

Mice that are homozygous for the *int-2* gene have been found to have defective inner ears that are poorly developed and devoid of endolymphatic ducts and spiral ganglion cells (Mansour *et al.*, 1993). The *Otx* genes (*Otx1* and *Otx2*) and the *Pax2* (paired box transcription factor) play an important role in the morphogenesis of the mouse inner ear (Morsli *et al.*, 1999; Burton *et al.*, 2004). Morsli and colleagues (1999), for example, observed a number of defects in the developing cochlea of *Otx1* 2/2 mutant mice (see Fig. 11.8). Examination on the inner ear of *Pax2* knockout mice has shown a wide Pax2 expression domain with anomalies in multiple regions of the cochlea (Burton *et al.*, 2004). The paint-filled inner ears of wild-type and *Pax2* mutants shown in Fig. 11.8 (Burton *et al.* 2004) illustrate the malformations in several portions of the developing mouse cochlea.

Mutations in the *Pax3* gene in humans may lead to Waardenburg syndrome, a condition characterized by disrupted melanocyte development, associated with cochlear impairment and sensorineural hearing loss (Redline *et al*., 1992; Tassabehji *et al*., 1992). The cause of the lethal effects of these mutations is not fully understood. A mutant human myosin gene, myosin VIIA which has been localized to a site on the long arm of chromosome 11, has been shown to cause both syndromic (in Usher syndrome) and nonsyndromic deafness (Petit, 1996; Steel & Brown, 1996). A human equivalent *diaphanous* gene of *Drosophila*, localized on chromosome 5, has been implicated in some cases of nonsyndromic deafness (Lynch *et al*., 1997). The *diaphanous* gene is believed to be responsible for actions of the cytoskeletal protein actin in the inner ear hair cells and their stereocilia. The gene connexin 26, which codes for a gap junctional protein that permits continuous electrical channels between neural elements, has been identified in the cochlea of rats (Lautermann *et al*., 1998). Mutations in connexin 26 cause hearing loss and in some cases deafness, possibly by interfering with K1 recycling in the sensory and nonsensory cells of the auditory epithelium (Zelante *et al*., 1997; Estivill *et al*., 1998). Identification of genes involved in the organogenesis of the mammalian ear is a noteworthy advance in our understanding of fetal development, and will have major impact on our clinical approaches to hearing impairment and deafness.

Eighth nerve and brainstem auditory neuronal development

The first-order neurons or spiral ganglion neurons of the eighth cranial nerve derive from neuroblasts in the otic cup and are associated with the neurogenic gene *Ngn1*. The auditory neuroblasts morph into the spiral ganglion medial to the otocyst. The auditory (rather than vestibular) identity of the neuroblasts is possibly initiated by the transcription factor Gata3, expressed in the neurogenic area of the otocyst and preserved in the spiral ganglion neurons (Fekete, 2009). Studies in knockout mice indicate that the basic helix-loop-helix transcription factor NeuroD is critical for neuronal differentiation of auditory spiral ganglion neurons. These neurons, which are bipolar in nature, innervate the receptors of the cochlea at one end, and form a tonotopic fiber of the eighth cranial nerve on the other projection from the soma. The developing neurons express the Trk receptors for BDNF and NT-3, which support innervation throughout the developing cochlea (Fekete, 2009).

The eighth cranial (auditory) nerve conveys the sensory cell information via 25 000 neurons from the cochlea to the brainstem. The eighth nerve spiral ganglion develops from the otocyst at around the twenty-eighth day of embryonic development. The bipolar cells migrate to the basement membrane and the epithelium of the otic vesicle to form the eighth nerve ganglion. This ganglion divides into two parts: (i) the pars inferior, which forms the cochlear nerve (this innervates the hair cells of the organ of Corti), and the vestibular nerve (this innervates the saccular macula of the posterior semicircular duct); and (ii) the pars superior (this develops into the vestibular nerve and innervates the utricular macula and ampullae of the lateral semicircular ducts). The myelinated central processes of the eighth nerve ganglion (first-order neurons) travel through the internal auditory meatus to synapse at the cochlear nucleus.

The cochlear nerve neurons bifurcate within the brainstem, sending an ascending tract to the anteroventral cochlear nucleus and a descending branch to the posteroventral cochlear nucleus. Second-order neurons of the higher auditory tract from the cochlear nucleus project to the ipsilateral lateral superior olive (near the midline of the ventral pons) and, through the trapezoid body, to contralateral structures of the auditory tract. The ascending auditory tract includes the superior olivary bundle and the lateral lemniscus. The medial superior olive receives ascending auditory neurons from bilateral cochlear nuclei, the medial superior olive cells receive projections from the contralateral cochlear nucleus, and the lateral superior olive is innervated by auditory fibers from the ipsilateral cochlear nucleus. The third order of auditory neurons arises in the brachium of the inferior colliculus. The fourth order of ascending auditory neurons arise in the medial geniculate nucleus, which sends auditory radiations to the primary auditory cortex (A1 and A2) in the temporal lobe (Brodmann's areas 31 and 32) (Kenna, 1990). Evidence suggests that early excitatory synaptic activity in the developing central auditory tracts and nuclei is necessary for normal function. Disruption of the development of structures in the auditory periphery or the central tracts and nuclei induces central hearing impairment (Moore, 1985, 1991; Kotak & Sanes, 1997).

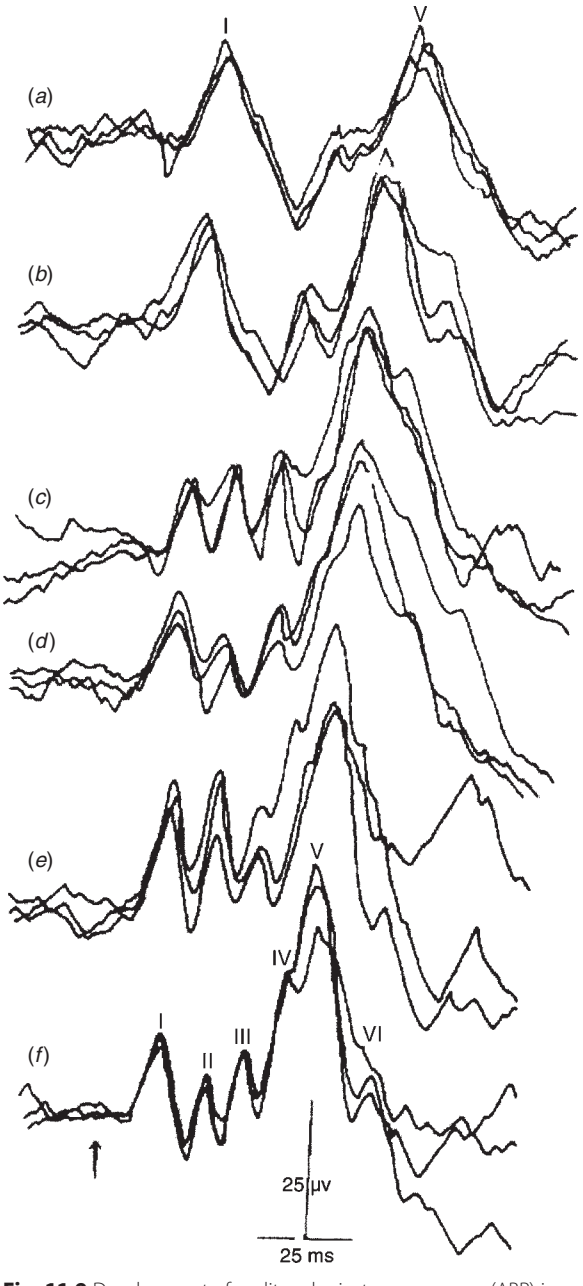


Fig. 11.9 Development of auditory brainstem responses (ABR) in normal neonates during the first year of development. The recordings show the absolute latencies of wave peaks I, II, III, IV, and V. The interpeak intervals (I–III, III–V, and I–V) indicate neural conduction times. Ages: (*a*) newborn, (*b*) 6 weeks, (*c*) 3 months, (*d*) 6 months, (*e*) 12 months, and (*f*) adult. (From Salamy & McKean, 1976 with permission.)

Both prenatal and postnatal studies of auditory-evoked responses indicate that the eighth nerve and higher auditory tracts and nuclei develop rapidly and continue to develop during the first few years of life. Noninvasive, scalp surface recorded auditory brainstem response (ABR) measures represent summated electrophysiological activity from the eighth nerve through the ascending tracts and nuclei of the pons and lower midbrain (Chiappa, 1997). The ABR typically consists of six prominent surface positive wave peaks, with wave V being the earliest and most conspicuous in the newborn. Observations of ABR recordings show that wave peak amplitudes increase and neural conduction times (as indicated by the interpeak latencies of neural responses) shorten as the gestation period progresses to the last prenatal week (Salamy & McKean, 1976; Starr *et al.*, 1977). Figure 11.9 shows ABR recordings in normal neonates during the first year of development which include wave peaks I, II, III, IV, and V. The interpeak intervals (I–III, III–V, and I–V) indicate neural conduction times. The I–III interval reflects the neuronal transmission from the auditory nerve to the lower pons, while the III–V interval indicates the transmission time from the lower pons to the midbrain. The I–V ABR interval latency represents the neural conduction time throughout the ascending auditory tracts and nuclei from the eighth nerve to the lower midbrain, and is generally around 5 ms in full-term neonates (Levy, 1997). These ABR waves may be used to screen newborns for early detection of hearing loss and brain damage. The maturation of the central and peripheral auditory systems continues, albeit at different rates, for the first years of life (Salamy, 1984). Congenital hearing loss may impair development in the auditory tracts and nuclei of the developing brain (Hardie, 1998; Hardie *et al.*, 1998).

Auditory function

Prenatal hearing

The functional development of the ear follows the maturation of the components of each of the three subsystems (outer, middle, and inner ear). It has been shown that the ear is functionally active by the fifth month of gestation when the fetus is capable of responding to internal and external sounds at that stage of development (Johansson *et al.*, 1964). At the twentieth week of gestation, the human fetus shows an increase in heart rate in response to externally generated tones delivered to the abdomen of the mother (Johansson *et al.*, 1964). During this period, the fetus has been bombarded with low-frequency sounds at around 85 dB (sound pressure level) in the amniotic fluid, including the mother's heartbeat, muscular

activities and possibly voice (Peck, 1995). Since sounds reaching the fetal ear in the amniotic sac are conducted by fluid (at a considerably higher velocity) rather than by air as in postnatal life, several acoustic features of the sound may differ. However, the fluid- and bone-conducted sound still reaches the functional ear of the fetus and may evoke a measurable physiological or motor reaction. Intrauterine startle responses to sound may be observed at week 28 of gestation. It is likely that the fetal ear is functionally capable of responding to a variety of biologically important sounds by week 28 of gestation.

Magnetic resonance imaging of the developing auditory system

Advances in magnetic resonance imaging (MRI) have added a new dimension to the study of the neuroanatomical and functional development of the central nervous system of the fetus (Gong *et al.*, 1998; Hykin *et al.*, 1999; Clements *et al.*, 2000; Lan *et al.*, 2000; Simon *et al.*, 2000; Chen & Levine, 2001; Chen *et al.*, 2002; Glenn & Barkovich, 2006a, b; Rutherford *et al.*, 2008). Functional MRI (fMRI) is effective in localizing the source of neuronal activity in the developing fetal brain in response to external sensory stimuli (Hykin *et al.*, 1999; Moore *et al.*, 2001; Fulford *et al.*, 2003, 2004). fMRI, which relies on natural hemodynamic changes in venous blood flow during neuronal stimulation, captures in vivo activation of specific brain centers in response to sensory input. Some fMRI studies have demonstrated unilateral temporal lobe sound-induced activity in the fetus at weeks 37–40 of gestation (Hykin *et al.*, 1999; Moore *et al.*, 2001). Moore and colleagues (2001) reported observation of the temporal and frontal lobe activation in 37- to 41-week-old fetuses in response to musical auditory stimulation of 85 dB SPL applied to the maternal abdomen. More recently, Jardri and colleagues (2008) reported measurable neuronal activity in the temporal lobe of fetuses at 33 weeks' gestational age in response to auditory stimulation of 0.5 kHz, 0.7 kHz, and 0.9 kHz at 94 dB SPL presented at the maternal abdomen. This study demonstrated that sound processing occurs in the auditory cortex of the temporal lobe as early as the beginning of the third trimester of pregnancy.

Neonatal hearing

At birth, the human auditory system is sensitive to airborne as well as to bone-conducted sound. The neonate is bombarded with ambient noises as well as the voices of the mother, father, and other caregivers. Auditory sensitivity in the neonate may be slightly less than that of older children with fully developed contiguous mechanical and neuroelectrical subsystems. Acoustic reflex startle reactions in response to loud sounds may be evoked in the first postnatal days (Downs, 1967; Northern & Downs, 1991). Newborns may be calmed by low-frequency sounds that accompany other stimulus-comforting modalities such as temperature and tactile sensation. The neonate can also discriminate sounds on the basis of frequency, intensity, and other acoustic features (Eisenberg, 1970). Downs (1967) found that sound could evoke the following behavioral responses in neonates: eye blink or eyelid reflexes, Moro's response, cessation of activity, limb movement, head turning, grimacing, sucking, arousal, breathing changes, and widening of eyes. These acoustic reactions were further categorized by Eisenberg (1970) to include overt reactions as follows:

- arousal;
- gross body movements;
- orienting behavior:
  - turning of head;
  - wide-eyed look;
  - pupillary dilation;
- motor reflexes:
  - facial grimaces;
  - displacement of a single digit;
  - crying or cessation of crying;
- cardiac reactions:
  - diphasic (deceleration–acceleration);
  - latency changes with various acoustic signals.

The normal infant is capable of sound localization in the environment at around two months after birth, and this skill becomes more refined over the next four months. At around six months, the infant can localize sound in the horizontal and vertical planes. Also, discrimination of ambient sound improves rapidly from birth, with the mother's voice probably being recognizable to the infant within the first month. At four weeks postnatally, the infant can discriminate phonemes, and at around 20 weeks is capable of "learning" phonemic contrasts. At 12 weeks, the infant can attend to the mother's voice (Northern & Downs, 1991).

Hearing acuity and speech sound discrimination continue to improve in the normal-hearing infant through the first three years. Evidence of hearing impairment in the infant may be detected at birth by objective ABR and otoacoustic emissions tests, and as early as six months by behavioral testing methods (Jacobson *et al.*, 1990). With advances in physiological hearing testing technology, universal newborn hearing screening has become feasible using auditory brainstem response and otoacoustic emissions techniques (Lutman, 2000). Otoacoustic emissions are essentially biological echoes of tonal stimuli that travel back from the cochlea through the middle ear to the ear canal, where they can be measured noninvasively by placing a small microphone at the entrance of the external auditory meatus. Otoacoustic emissions originate in the outer hair cells of the cochlea and reflect the integrity of the inner ear. Early identification of hearing loss in infants permits early intervention and possible corrective measures (Kemp, 1978; Downs & Yoshinaga-Itano, 1999; Yoshinaga-Itano, 2000).

Higher auditory functions include the ability to localize sounds in space and speech discrimination. These abilities develop in early postnatal life in the normal neonate. Speech discrimination of phonemes and suprasegmentals (needed for normal speech and language development) is well developed around the tenth month of the postnatal period. Auditory experience and learning are important aspects of neonatal hearing development. Several features of experiential development are relevant to auditory development (Werker, 1989; Peck, 1995):

- maturation or normal development of the auditory system;
- maintenance or the activation and normal use of the auditory system;
- facilitation or enabling perception and auditory experience;
- tuning or making the perceptual mechanisms more refined or sharper;
- induction or experientially based recognition and response.

MRI studies in neonates have demonstrated functional activation of the cortex in 3-month-old infants in response to auditory native speech stimuli (Dehaene-Lambertz *et al.*, 2002). Using fMRI of the brains of 3-month-old infants, Dehaene-Lambertz and colleagues found lateralized left cortical activation in the superior temporal and angular gyri in response to speech stimuli, similar to that observed in adults. These findings are consistent with electrophysiological studies using event-related evoked potentials and behavioral studies that show activation of the temporal lobes in response to speech sounds (Pannekamp *et al.*, 2006; Homae *et al.*, 2007).

Assessment of hearing in the neonate

In 1995, the American Academy of Pediatrics (AAP) endorsed the position of the Joint Committee on Infant Hearing (JCIH), which supported the aim of "universal detection of hearing loss in infants before three months of age, with appropriate intervention no later than six months of age" (AAP Joint Committee on Infant Hearing, 1995). This and other related pediatric initiatives led to the concept of universal newborn hearing screening, which is widely used today in the United States and Europe (Wessex Universal Neonatal Hearing Screening Trial Group, 1998). The following extract is taken from the report of the AAP JCIH, 1995:

> The Academy recognizes that there are five essential elements to an effective universal newborn UNHSP: (1) initial screening, (2) tracking and follow-up, (3) identification, (4) intervention, and (5) evaluation. The child's physician and parents, working in partnership, make up the child's medical home and play an important role in each of these elements of a UNHSP.
>
> Guidelines for the screening element of a UNHSP:
>
> - Universal screening has as its goal that 100% of the target population, consisting of all newborns, will be tested using physiologic measures in both ears. A minimum of 95% of newborns must be screened successfully for it to be considered effective.
> - The methodology should detect, at a minimum, all infants with significant bilateral hearing impairment, i.e., those with hearing loss ≥ 35-decibel in the better ear.
> - The methodology used in screening should have a false-positive rate, i.e., the proportion of infants without hearing loss who are labeled incorrectly by the screening process as having significant hearing loss, of ≤ 3%. The referral rate for formal audiologic testing after screening should not exceed 4%.
> - The methodology used in screening ideally should have a false-negative rate, i.e., the proportion of infants with significant hearing loss missed by the screening program, of zero.

- Until a specific screening method(s) is proved to be superior, the Academy defers recommendation as to a preferred method. Currently, acceptable methodologies for physiologic screening include evoked otoacoustic emissions (EOAE) and auditory brainstem response (ABR), either alone or in combination. Both methodologies are noninvasive, quick (<5 minutes), and easy to perform, although each assesses hearing differently. EOAE measures sound waves generated in the inner ear (cochlea) in response to clicks or tone bursts emitted and recorded via miniature microphones placed in the external ear canals of the infant. Although EOAE screening is even quicker and easier to perform than ABR, EOAE may be affected by debris or fluid in the external and middle ear, resulting in referral rates of 5% to 20% when screening is performed during the first 24 hours after birth. ABR measures the electroencephalographic waves generated in response to clicks via three electrodes pasted to the infant's scalp. ABR screening requires the infant to be in a quiet state, but it is not affected by middle or external ear debris. Referral rates <3% may be achieved when screening is performed during the first 24 to 48 hours after birth. Referral rates <4% are generally achievable with EOAE combined with automated ABR in a two-step screening system or with automated ABR alone. In a two-step system using EOAE as the first step, referral rates of 5% to 20% for repeat screening with ABR or EOAE may be expected. The second screening may be performed before discharge or on an outpatient basis within 1 month of age. Screening should be conducted before discharge from the hospital whenever possible.
- Each birthing hospital should establish a UNHSP with a designated medical (physician) director and sufficient staff to perform the screening tests.

In 2007, the JCIH issued an updated position statement on universal newborn hearing screening that included a number of important modifications of standard protocols in the JCIH 2000 statement (American Speech-Language-Hearing Association, 2007). This statement is presented here, with permission:

Current Challenges, Opportunities, and Future Directions

EHDI programs throughout the nation have demonstrated not only the feasibility of universal newborn hearing screening but also the benefits of early identification and intervention. There is a growing body of literature indicating that when identification and intervention occur no later than six months of age for newborn infants who are deaf or hard of hearing, the infants perform as much as 20–40 percentile points higher on school-related measures (vocabulary, articulation, intelligibility, social adjustment, and behavior). Still, many important challenges remain. Despite the fact that approximately 95% of newborn infants have their hearing screened in the United States, almost half of newborn infants who do not pass the initial screening fail to have appropriate follow-up to confirm the presence of a hearing loss and/or initiate appropriate early intervention services. Despite the tremendous progress made since 2000, there are many challenges to the further development of successful EHDI systems. Many of these challenges, opportunities for system development, and areas for research are outlined in the complete JCIH 2007 Position Statement. The critical need for training professionals with pediatric-specific and discipline-appropriate knowledge and skills to work with infants, children, and families in EHDI programs is also addressed.

Conclusion of the JCIH

Since the JCIH 2000 statement, tremendous and rapid progress has been made in the development of EHDI systems as a major public health initiative. The percentage of infants screened annually in the United States has increased from 38% to 92%. The collaboration at all levels of professional organizations, federal, and state government, hospitals, the medical home, and families has contributed to this remarkable success. New research initiatives are continuing to develop more sophisticated screening and diagnostic technology, digital hearing aids and FM systems, speech-processing strategies in cochlear implants, and optimal intervention methods. It is apparent, however, that there are still serious challenges to be achieved and system barriers to be conquered to achieve optimal EHDI systems in all states in the next five years. We must never lose sight of our ultimate goal to optimize the communicative, social, academic, and vocational outcomes of every single child with permanent hearing loss.

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Chapter 12

Newborn behavior and perception

Elena V. Kushnerenko and Mark H. Johnson

Introduction

Traditionally, the behavior of human newborns has been considered to be essentially reflexive over the first few weeks of life. However, more recently evidence has accumulated to indicate that newborns are already well equipped to acquire and learn information rapidly. In addition, through prenatal learning and other mechanisms, newborns are biased to orient and attend to specific types of information that will become of high relevance to their future learning and survival. In this chapter, we begin by outlining evidence for the learning abilities of newborns, before discussing some domains for which there is evidence that newborns have specific biases or abilities.

Two abilities that are already present at birth are orienting to novelty and habituation to repeated stimuli. These processes can be revealed by registering changes in newborns' looking and sucking behavior. Cardiac and brain activity measures, when incorporated in careful experimental design, may also shed light on newborns' brain function.

Orienting

Orienting towards new stimuli is not only essential for the survival of any organism, it is also a key prerequisite of learning. Therefore, it is of primary importance for neonates. The orienting reflex is a combination of overt and covert responses associated with searching for and preferential processing of new information (Pavlov, 1927; Sokolov, 1963; Sokolov *et al.*, 2002). Components of the orienting reflex are targeting responses (eye and hand movements), autonomic reactions (cardiac and skin conductance response), desynchronization of the electroencephalogram, and augmentation of certain event-related potential (ERP) components (Sokolov *et al.*, 2002; Kushnerenko *et al.*, 2007). In accordance with its primary biological significance, the orienting reflex appears very early during ontogenesis (for a review, see Gomes *et al.*, 2000). Cardiac orienting reflex has also been documented in near-term fetuses (Lecanuet *et al.*, 2000).

In infants, orienting is most commonly assessed on the basis of spontaneous motor and psychophysiological responses, e.g., localized head turning (Clarkson *et al.*, 1989; Morrongiello *et al.*, 1994) or changes in heart rate (Clarkson & Berg, 1983). Sometimes such parameters as behavioral inhibition, motor quieting and eye widening are also used in assessing newborn's responsiveness (Gomes *et al.*, 2000).

A newborn's responsiveness varies depending on parameters of stimuli: newborns preferentially respond to broadband noise than to tones (Turkewitz *et al.*, 1972; Werner & Boike, 2001; Kushnerenko *et al.*, 2007); to high-frequency than to low-frequency noise (Morrongiello & Clifton, 1984); to tones of longer duration than of shorter duration (Clarkson *et al.*, 1989); and to pulsed than to continuous signals (Clarkson & Berg, 1983). The latter may be of particular importance, since sensitivity to temporal transitions is important for speech discrimination. Physical features of the stimuli (such as intensity or spectral complexity) may initially be preferred by newborns as the most salient cues for orienting (Kushnerenko *et al.*, 2007). Interestingly, newborns are able to match stimuli across modalities (auditory and visual) according to their intensity (Lewkowicz & Turkewitz, 1980), an ability that adults do not appear to be capable of.

During the first months of life, an infant's responsiveness is influenced by their previous experience, and infants orient more to novelty (Gomes *et al.*, 2000) than to physical features of the stimuli. This developmental specialization and narrowing of the

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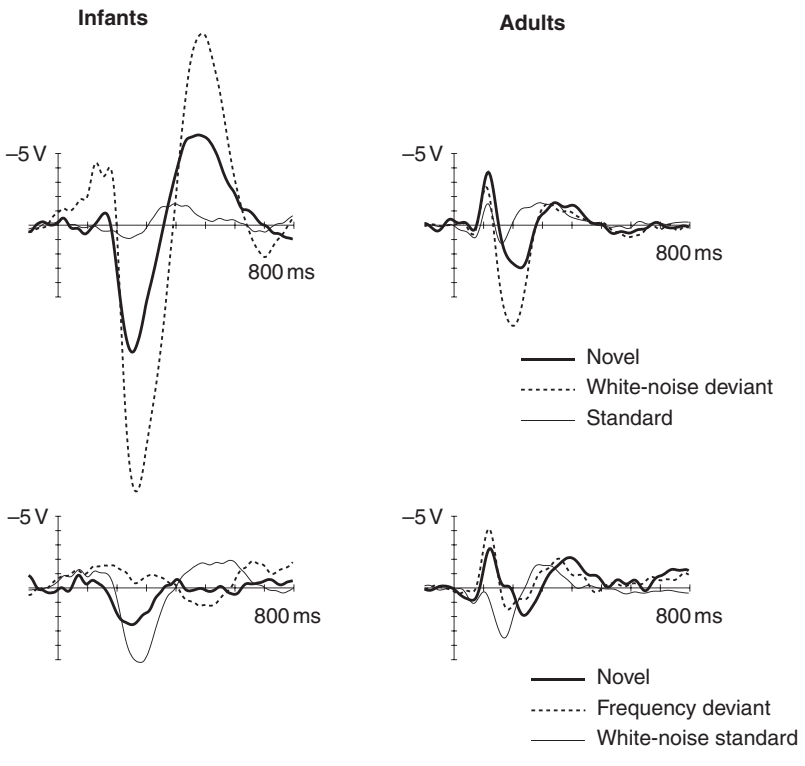

Fig. 12.1 Event-related potentials (ERPs) elicited in response to tones and broadband sounds in newborns and adults in an oddball paradigm. In both, infants (*left side*) and adults (*right side*), the ERP response consists of the early negative peak first followed by a positive wave and then by a broad negativity.

Upper row: the repetitive sequence of a harmonic tone (standard, thin continuous line) was occasionally broken either by a white-noise segment (dotted line) or by various environmental (novel) sounds (thick continuous line). Observe the large amplitudes of all three components in response to white noise in newborns.

Bottom row: the repetitive sequence of a white-noise segment was occasionally broken either by a harmonic tone or by various environmental (novel) sounds. In newborns, no significant difference was found between the responses elicited by the white-noise standards and "novels," therefore the high amplitude in response to "novels" in newborns is due to spectral richness of the stimuli and not novelty per se. In adults, both novels and deviants elicited significant early negative responses in both conditions indicating that the "novelty" of the stimuli has been detected.

Note that the early negativity in newborns is only observable in response to large spectral change (harmonic tone vs. white-noise). (Adapted with permission from Kushnerenko *et al.*, *European Journal of Neuroscience*, © 2007, Wiley-Blackwell Publishing.)

broadly tuned perception in infants probably helps the development of selective attention and reduces distraction to irrelevant stimuli (for a review, see Gomes *et al.*, 2000).

Kushnerenko and colleagues (2007) have shown that broadband noise sounds elicited unexpectedly high-amplitude and reliable ERPs in newborns, whereas in adults the response to this stimulus was not much larger than to the other stimuli tested (Fig. 12.1). In adults, sound energy and spectral differences showed much smaller effects, whereas contextual changes were processed faster, compared with newborns. These results suggest that maturation and learning lead to faster and more elaborate processing of higher-level cognitive constructs.

Habituation

As a stimulus loses its novelty with repeated presentations, the size of the orienting response decreases, a process called habituation. Habituation of the orienting reflex during conditioning was described by Sokolov (1963). In Sokolov's model, an internal representation (engram) of a stimulus is formed each time it is encountered, and the extent to which the external stimulus matches that representation determines how long subjects attend to it. Once the representation is complete, that is the stimulus contains no more new information, the subject no longer pays attention to it. Thus, habituation is an adaptive mechanism which serves to eliminate responses to biologically irrelevant stimuli. However, once a stimulus is perceived as different from the "engram," the orienting response will be elicited again, a phenomenon called recovery of the orienting response, or dishabituation.

Habituation procedures are used to test whether infants can discriminate stimuli from two categories. The stimulus from the first category is presented only when the infant is sucking at high intensity (high-amplitude sucking [HAS]) or looking at the visual target. These behaviors are indicative of infants' heightened attention and the stimuli themselves serve as conjugate reinforcers. As an infant loses interest in the repeated presentation, the performance of the learned behavior decreases, and habituation occurs.

The stimulus from another category is presented when the rate of sucking or looking response drops below the preset criterion. Looking at the visual target is used in habituation procedures both in auditory and visual preference procedures – the looking time to target is measured while stimuli from two different categories are presented.

Instrumental conditioning

In addition to learning in habituation procedures, newborns have also shown the ability for instrumental conditioning (for a review, see Moon & Fifer, 2000). Since sucking can be reliably elicited in most infants during the first days of life, DeCasper and Fifer (1980) developed an instrumental conditioning procedure using a non-nutritive sucking preference to assess the newborn's perceptual and learning capacities. In this procedure, 1- to 4-day-old infants learn to activate and maintain different sounds presented by adjusting the duration of their sucking bursts or the pauses between sucking bursts.

This research demonstrates that neonates within the first days of their life are able to rapidly learn to control their behavior. Further, by the age of 8–10 weeks infants have been shown to operate their rate of kicking in a mobile conjugate reinforcement procedure (Rovee & Rovee, 1969). In this procedure, an infant's kicks move a crib mobile via a ribbon attached to the mobile and to the infant's ankle. Infants learn to operate this system within just a few minutes. The increase in kicking rate could not be due to a general increase in arousal on seeing a mobile, since infants in the control group, who saw this mobile moving noncontingently, did not increase their rate of kicking. In recent studies, the mobile procedure has also been used to assess the long-term memory of infants (for a review, see Rovee-Collier, 1997).

Behavioral learning techniques are not always easy to apply due to the poor ability of newborns to perform a coordinated motor act and to maintain a certain level of attention and arousal. The level of responsiveness in newborns depends largely on their state of arousal. Wolff (1966) found that babies in the state of alert inactivity reacted to stimulation by being more active, whereas those in an active state seemed to do the opposite and calm down. In contrast, some electrophysiological paradigms allow us to obtain information on brain functioning independently of the newborn's state and without requiring an active response or attention from the infant. For example, the electrophysiological correlates of auditory information processing can be recorded in newborn infants even in sleep, which is very convenient since sleep is the dominant state in newborns.

Electrophysiological responses

A link between rapidly emerging psychological and behavioral functions and the underlying neural mechanisms can be provided by investigating the electrical activity generated by neurons within the functioning brain. The electrical signals related to some external or internal event (ERPs) provide real-time indices of neural information processing, and can be studied throughout the crucial period of our most rapid neuroanatomical and functional development – early infancy.

The most-promising ERP paradigm used in research of auditory information processing in newborns is the so-called "oddball" paradigm and its variations (Figs. 12.1 and 12.2). Sequences of homogeneous repeated "standard" stimuli are randomly and infrequently interrupted by a different "deviant" stimulus. The electrophysiological response to this "deviation" is visualized by subtraction of the evoked potentials to standards from that to deviants, resulting in a negative difference wave voltage (in adults), accordingly called the Mismatch Negativity ([MMN], Näätänen *et al.*, 1978). The mechanism underlying the MMN is thought to be based on memory trace formation for the repeated stimulus (neural representation of the stimulus, see also above-mentioned Sokolov's "stimulus engram" [Sokolov, 1963]), and further automatic comparison of whether each new stimulus "matches" or "mismatches" this memory trace. Recording mismatch responses is a feasible way to assess the newborn's auditory discrimination abilities, since it can be recorded even in the sleeping neonate. However, the polarity and latency of an infant's mismatch response seem to be largely dependent on the parameters of auditory stimuli (Kushnerenko *et al.*, 2007; Csibra *et al.*, 2008) (see Figs. 12.1 and 12.2), and on the infant's age (Kushnerenko *et al.*, 2002; Morr *et al.*, 2002; Leppanen *et al.*, 2004).

Unlike in adults, the state of arousal is not crucial in infant auditory electrophysiological studies: newborns' auditory mismatch responses recorded during quiet or active sleep or during awake state do not essentially differ from each other (Kushnerenko, 2003). Recent electrophysiological studies reported that newborn infants are even capable of learning

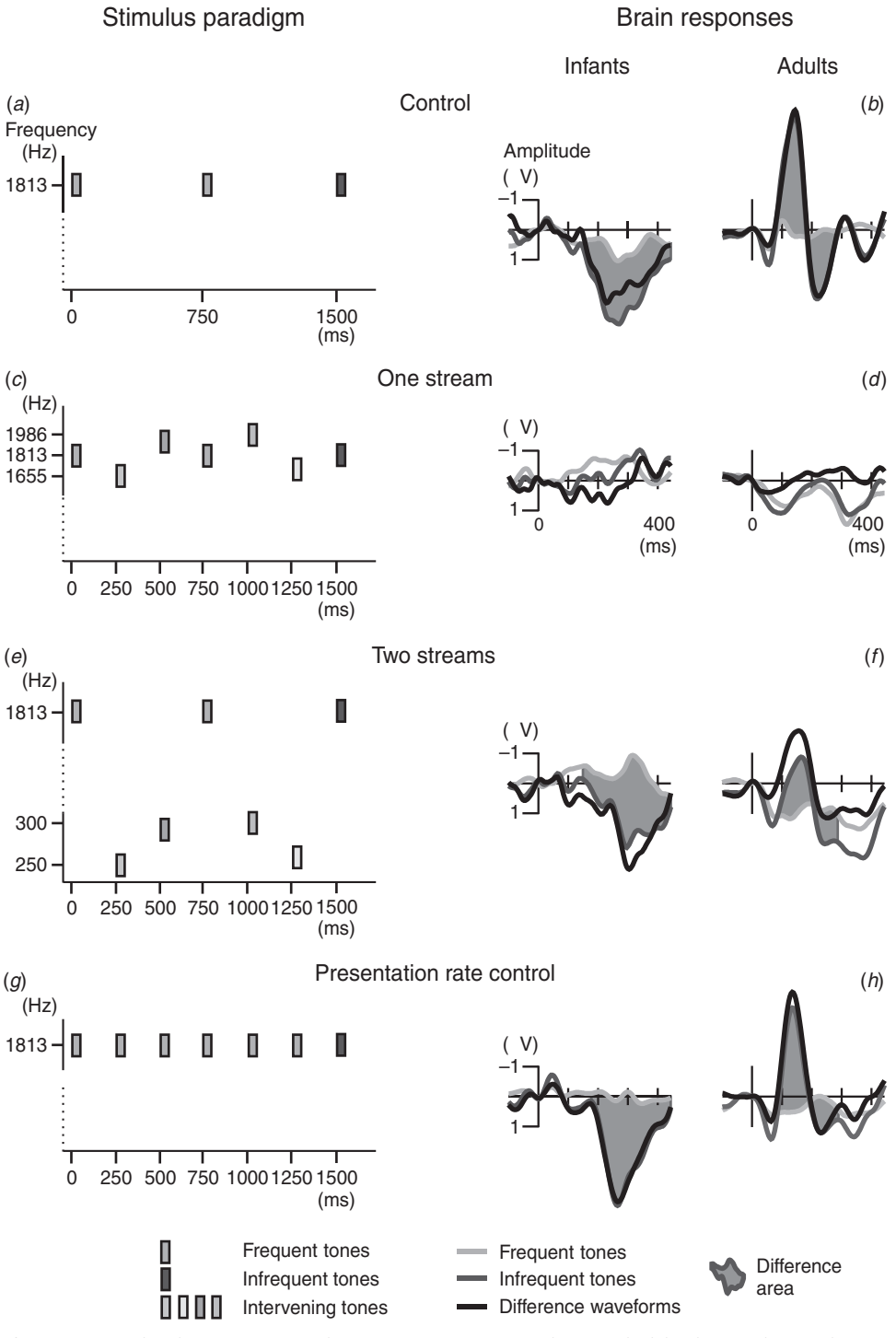

Fig. 12.2 (See color plate section.) Sound streams segregation in newborns and adults: the stimulus paradigm and electric brain responses. (*a*) Schematic illustration of a segment of the control-condition sequence. Rectangles represent tones; their y-axis coordinate shows the tone frequency (logarithmic scale). Different loudness-level settings are marked with different colors: pastel blue, frequent soft (standard) tones; dark red, infrequent louder (deviant) tones. (*b*) Control-condition responses. Frontal (F4) electric brain responses (*left*, infants; *right*, adults) elicited by the standard (pastel blue lines) and deviant (dark red line) tones together with their respective difference waveform (black line). The light red shading of the area between the standard and deviant responses marks significant differences between the two brain responses. (*c*) In the one-stream condition, intervening tones varied in frequency (shown by the y-axis position of the rectangle) and intensity (marked with different colors).

while they are asleep (Cheour *et al.*, 2002; Ruusuvirta *et al.*, 2003; Carral *et al.*, 2005). These results support functional differences between infant and adult sleep. Indeed, although newborns spend almost 20 hours per day in sleep, they are still able to learn and develop very rapidly.

Meeting mother

The first hour or two after birth may represent a "sensitive period" for mothers and infants to enable mother–infant bonding and attachment. Within minutes after birth, newborns placed naked on the mother's bare skin between or below her breasts (skin-to-skin contact) displayed organized "prefeeding behavior," (for a review, see Winberg, 2005). This behavior is first expressed by rooting and sucking and then by locating the nipple without assistance and beginning to suck. In a study by Varendi and Porter (2001), 3-day-old neonates demonstrated a kind of "crawling" activity of the arms and strong pushing with the legs, trying to move towards their mother's breast odor. The newborns were positioned on a warm cot, and a cotton pad worn by their mother against her areola for a couple of hours was put 17 cm in front of them. By pushing with their legs, 18 babies out of 22 reached the breast pad and only 3 reached the clean pad. Importantly, these three babies reached their mother's pad too in the first part of experiment (all babies were tested twice, randomly assigned to be tested either with breast pad or clean pad first). Winberg (2005) suggested the existence of "innate central instructions, which activate locomotion generating centers after appropriate sensory stimulation."

Caption for Fig. 12.2 (cont.) (*d*) One-stream condition responses. The responses to the standard and deviant tones did not significantly differ from each other in either group of subjects. (*e*) For the two-stream condition, the frequencies of the intervening tones were lowered from the values used in the one-stream condition (see y-axis positions), but the tone intensity values were retained (see rectangle colors). (*f*) Two-stream condition responses. The responses to the standard and deviant tones significantly differed from each other in both groups of subjects, and they were similar to those elicited in the control condition. (*g*) In the presentation-rate control experiment, "intervening" tones were identical to the frequent tones (same frequency and same intensity). (*h*) Presentation-rate control experiment responses. The responses to the standard and deviant tones significantly differed from each other in both groups of subjects and were similar to those elicited in the control and two-stream conditions of the main experiment. Note the positive polarity of the newborns' mismatch response in this study. (Reprinted with permission from Winkler *et al.* (2003), © 2003 *National Academy of Sciences of the U S A.*)

Within a few days newborns began to feed with more confidence and to find the nipple more easily. The mother's breast odor was shown to be the crucial and su cient cue to guide newborns in their first attempts to reach the nipple (Varendi *et al.*, 1994; Varendi & Porter, 2001). These results, together with studies reporting newborns' preference for mother's breast odor over other female odors (MacFarlane, 1975; Kushnerenko *et al.*, 1998, 1999), raises the question of whether this preference was formed by early postnatal experience, or in the prenatal environment. There are studies supporting both these views. The prenatal exposure to amniotic fluid may prime newborns with maternal chemical cues (Varendi *et al.*, 1996). In the study by Varendi and colleagues (1996), neonates were given a choice between the mother's breast moistened with her amniotic fluid and an untreated breast; 23 of 30 infants preferred the breast treated with amniotic fluid. A subsequent study by the same authors has shown that olfactory learning in infants occurs within minutes after birth (Varendi *et al.*, 2002). Neonates merely exposed to an odor for 30 minutes within the first hour after birth spent more time turning to the familiar exposure odor than to a novel one.

The above results may be of particular importance for understanding early mother–infant bonding. Within the first one to two hours immediately after birth, healthy vaginally delivered infants are typically highly alert and responsive to stimulus input. Vaginal delivery elicits a strong activity of the sympathetic system (Lagercrantz & Slotkin, 1986) and results in increased levels of catecholamines, in particular norepinephrine, as well as locus ceruleus activation. This activation may account for the alert state and possibly heightened learning e ciency shortly after birth. In mammalian models of imprinting hyperfunctioning, norepinephrinergic locus ceruleus is believed to support olfactory learning during the neonatal sensitive period (for a review, see Sullivan, 2003). A recent study of human newborns has shown that heightened olfactory learning was found in neonates with higher norepinephrine plasma levels (in a group that experienced labor contractions before cesarian section) (Varendi *et al.*, 2002). Since in an uncomplicated delivery, the odor of the mother is the first odor that a newborn encounters, enhanced olfactory learning within a short time after birth may have a positive effect on early mother–infant interactions.

Several studies have also demonstrated mother's voice recognition within the first days after birth

(DeCasper & Fifer, 1980; Deregnier *et al.*, 2000). DeCasper and Fifer (1980) showed that newborns younger than 3 days of age when tested in the non-nutritive sucking preference procedure learned to adjust their sucking to listen to different voices. The mothers' voices were tape-recorded shortly after delivery, and presented to their newborns, depending on their sucking temporal properties. The newborns learned rapidly to gain access to their mother's voice and worked to produce their mother's voice in preference to those of other females. These results indicate that newborns are able to discriminate voices, with the mother's voice being most e cient as a reinforcer. Since these infants were housed in maternity hospital nurseries, they had only limited maternal exposure. Thus, the authors suggested that their data are indicative of prenatal learning. A mother's voice is the most intense acoustical signal available to the fetus, although, due to low-pass filtering properties of the intrauterine environment, it may be mu ed (Abrams & Gerhardt, 2000). Prosodic cues, such as pitch contours, speech rhythm, and stress are, however, clearly represented in intrauterine tape recordings (Gri ths *et al.*, 1994), and therefore can be learned by the fetus.

The newborn's preference for their mother's voice may result also from some early postnatal learning shortly after birth, or at least this explanation is rarely completely ruled out. However, there is one experiment that directly tested prenatal auditory learning. DeCasper and Spence (1986) asked women to read aloud a short speech passage two times a day during the last six weeks of pregnancy. After their babies were born, the infants participated in the non-nutritive sucking preference procedure. Two stories were read to the infants, one that they were exposed to while in the womb and another one they had never heard before. Newborns preferred to listen to the passage that they had heard prenatally (DeCasper & Spence, 1986). Thus, the child's attachment to the mother may start even before birth through learning some aspects of mother's voice and odor. This learning may be further consolidated following birth by rapid acquisition of additional mother cues. Early olfactory and auditory competence probably serves to help newborns to recognize their mother.

Having a "sensitive period" for forming an attachment may appear maladaptive for human beings, but nevertheless, early skin-to-skin contact shortly after birth was shown to be extremely important for mutual regulation of physiology and behavior between mother and newborn (for a review, see Winberg, 2005) and to quickly counteract "the stress of being born" when adaptive effects of catecholamine surge are over, presumably decreasing sympathetic and increasing parasympathetic tone (Bystrova *et al.*, 2003).

Vision, faces, and social interaction

Of all the sensory modalities, vision is perhaps the most dominant in humans. While newborns have relatively poor visual acuity (Atkinson, 2000), this is su cient to detect objects and faces presented within around a meter or so. A particularly active area of research over past decades has concerned newborns' responses to the faces of other human beings. A study published in 1991 replicated earlier reports that human newborns preferentially orient towards simple schematic face-like patterns (Johnson *et al.*, 1991). The finding that infants possess some information about the characteristics of faces from birth has come under continuing scrutiny over the past decade (for a review, see Johnson, 2005a). The early experiments with newborns indicated that a pattern containing high-contrast blobs corresponding to the approximate location of the eyes and mouth might be su cient to elicit the newborn preference. This stimulus is reminiscent of the low spatial frequency view of a face that the newborn visual system may allow (Fig. 12.3).

More than 16 studies of face-related behavior in human newborns have been published since 1991 (for a review, see Johnson, 2005a). Although most of these papers concluded that newborns are biased to attend to stimuli that possess certain characteristics of faces, two alternative views have been expressed. The first of these alternative views, the sensory hypothesis, is that all newborn visual preferences, including those for face-related stimuli, can be accounted for simply in terms of the relative visibility of the stimuli. The newborn visual system is restricted to the lower part of the range of spatial frequencies that is visible to adults (low spatial frequency information). It has been proposed that newborns prefer to look at faces merely because the amplitude at different frequencies of these stimuli happens to best match the sensitivity of the newborn visual system. The sensory hypothesis has fallen out of favor because, even when amplitude is controlled, phase information (configuration) still influences the newborn preference toward faces.

The second alternative view is that newborns have complex face processing abilities. The findings used to support this claim include a preference for images of attractive faces, data indicating that newborns are sensitive to the presence of eyes in a face, and evidence

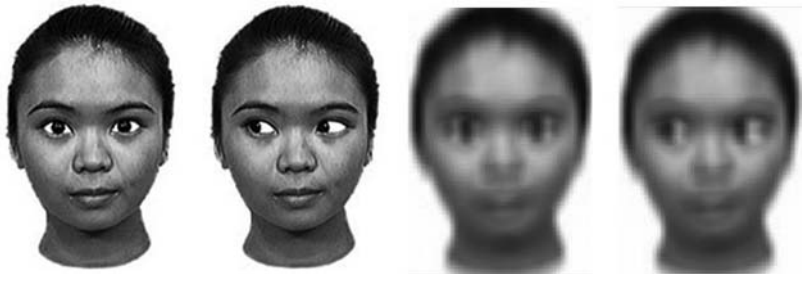

Fig. 12.3 A low spatial frequency view of a face as it may be visible to a newborn.
Left: Realistic images of faces as viewed through the visual system of newborns at a typical distance for face-to-face interaction (approximately 50 cm).
Right: The same realistic images of faces as viewed through the visual system of newborns, but from approximately 2 m distance. Viewed from at a distance, or in the periphery, the configuration of shadowed areas characteristic of a naturally lit face may also activate the subcortical route.
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that they prefer to look at faces that engage them in eye contact (Farroni *et al.*, 2002). However, as we will discuss shortly, we believe that all of these results could be accounted for by a low spatial frequency face configuration detector. For example, older infants prefer more attractive faces because these faces are closer to an average or prototypical face. Inspection of realistic face images through the appropriate spatial frequency filters for newborns reveals that a mechanism that is sensitive to the configuration of a face could be preferentially activated by: the most prototypical face configuration presented; the presence (or absence) of open eyes; and direct versus averted gaze.

Despite the alternative views, most authors agree that the stimulus preferences observed in newborns are sufficient to elicit orienting toward real faces in the natural visual environment of the newborn. The current prevailing view on the mechanisms that underlie the preference of newborn babies for face-like stimuli is that newborns have one or more biases in visual processing that are sufficient, in their natural environment, to ensure that they fixate faces. Johnson and Morton (1991) proposed that a stimulus equivalent to the low spatial frequency components of the configuration of a face is optimal for eliciting the preference (see Fig. 12.3). Recently, it has been proposed that the configuration of high-contrast areas associated with the eyes and mouth is not required, but that newborns might prefer up-down asymmetrical patterns with more elements or features being contained in the upper half of a bounded object or area (Turati *et al.*, 2002). Although such preferences are sometimes said to be due to several "domain-general" biases, such as a possible upper visual field bias, experiments indicate that there is a crucial interdependency between the borders of the stimulus and the elements within it, indicating some complexity to the bias. Experiments that independently manipulate upper visual field elements and bounded areas, and experiments that measure eye movements sufficiently to control upper/lower visual field presentation, have not yet been published.

In addition to the factors noted above, recent experiments suggest that phase contrast of stimuli is also important for newborns' preferences (Farroni *et al.*, 2005). In these experiments newborns showed a preference for an upright face (with both schematic and naturalistic images) only under positive (face-like) contrast conditions. If phase contrast is added to the previous requirements for the "top heavy bias" underlying newborn face preference, it is clear that a more complex representation is required than just an upper visual field bias.

When recent evidence is considered we are left with two candidate stimuli that could best elicit newborn face-related preferences. One of these is a raised surface or area with more indentations or dark areas in the upper half, while the other involves indentations or darker blobs corresponding to the approximate locations of eyes and mouth (Fig. 12.4). At a distance, or in the periphery, a mechanism activated by these stimuli would direct attention toward faces. When closer to the infant, the same mechanism may direct attention to the eyes of a face.

The brain basis of newborn visual preferences

Although there is an increasing literature on the neural basis of face detection in human infants of 2 months

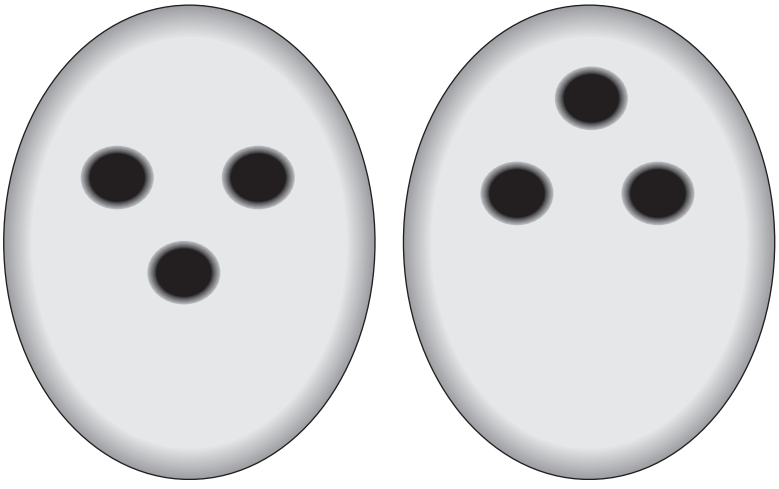

Fig. 12.4 The optimal stimuli for newborn preferences. These hypothetical representations were created by putting together the results of a number of experiments on newborns' face-related preferences. Conclusions were combined from experiments showing the importance of the number of elements in the upper half of a bounded area or surface, the importance of a face-relevant pattern of phase contrast, and the importance of the basic face configuration as viewed at low spatial frequency. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Neuroscience*, Johnson, 2005b, © 2005.)

and older (for a review, see de Haan *et al*., 2002), for a variety of technical and ethical reasons it has not yet proved possible to use functional magnetic resonance imaging, magnetoencephalography, or positron emission tomography to study face perception in healthy newborns. However, a number of converging lines of evidence support the view that face-related looking behavior in newborns is mediated largely by a subcortical visuomotor route. First, neuroanatomical, functional imaging, electrophysiological, and behavioral evidence indicates that while visual cortical areas can be partially activated in newborns, they are relatively immature. Further, the partial activation of visual cortical areas in the first months may have little influence over the visually guided behavior of the infant (Csibra *et al*., 2000). Compared with the cortical visual route, structures on the subcortical route seem to be more developed around the time of birth (for a review, see Johnson, 2005a). A second line of evidence supporting the view that newborns' orienting to faces preference is mediated by a subcortical route comes from other species. Almost all vertebrates can recognize biologically relevant stimuli at, or shortly after, birth. In avian, rodent, and primate species, a subtelencephalic route is involved in innate stimulus recognition (Sewards & Sewards, 2002).

As the nasal and temporal visual fields feed differentially into the cortical and subcortical visual routes in humans, it is possible to gain converging evidence for subcortical processing by presenting stimuli in either the temporal or nasal visual fields only. Specifically, stimuli presented in the nasal field differentially feed into the cortical route, while those in the temporal field feed into the subcortical route. In one experiment, newborns wore patches on one eye while face-like stimuli were presented to the other eye in either visual field. Consistent with the view that face preferences in newborns are due to the action of subcortical processes, the face-related preference was observed only when stimuli were presented in the temporal visual field (Simion *et al*., 1998).

Finally, much recent evidence from functional imaging and neuropsychology with adults indicates the presence of a subcortical route for face detection (see Johnson, 2005b). The converging lines of evidence discussed above support the hypothesis that the adult subcortical face route functions in newborns, and is responsible for the patterns of face-related stimulus preference that are seen at that age. This working hypothesis implies that the subcortical route not only detects the presence of faces, and orients the newborn toward them, but also might activate relevant cortical regions such as the lateral occipital, fusiform, and orbitofrontal cortices. Indeed, it is possible that the projection pattern to the cortex from the subcortical route partly determines which cortical regions become incorporated into the social brain network during development (Johnson, 2005b). If the subcortical pathway operates from birth in humans, then it could enhance the activity of cortical areas that, at that stage, receive only poor input through the cortical visual route. Such early enhancement of activity in selected cortical areas, together with other architectural biases, might assist in the recruitment of these specific cortical areas for face processing and social cognition.

Discovering language

Another early capacity newborns demonstrate shortly after birth is their speech preferences. Newborns prefer to listen to speech than to nonspeech analogs (Vouloumanos & Werker, 2007a) and to their native language rather than a foreign language (Mehler *et al.*, 1988; Moon *et al.*, 1993). However, native language preference depends on the two languages presented being from distinct language families, and being quite different rhythmically (Nazzi *et al.*, 1998). For example, French newborns can discriminate English from Japanese, but not from German, which is rhythmically more similar (Nazzi *et al.*, 1998). Further, the cues for discrimination between languages are not present in backward speech. Ramus and colleagues (2000) used a habituation-dishabituation procedure to show that both human newborns and tamarins can discriminate sentences from Dutch and Japanese but not if the sentences are played backward. In this regard it is noteworthy that brain imaging studies in neonates and three-month-olds have revealed that activation in a speech-related area of the cortex (left planum temporale) reached significance only for forward but not for backward speech (Dehaene-Lambertz *et al.*, 2002; Pena *et al.*, 2003), even though backward speech shares common segmental features with forward speech. Newborns appear to use prosodic cues to distinguish one language from another and these cues are disturbed in the backward speech.

An intriguing finding is that newborns prefer to listen to the language they heard while in the womb (Moon *et al.*, 1993). These results suggest that some acquaintance with native language occurs either prenatally or immediately after birth. While in the womb infants may learn some prosodic regularities of the language that are available in utero being transmitted through the mother's body (Gri ths *et al.*, 1994). However, newborns are also capable of discriminating fine phonetic differences, for example between two vowels (Clarkson & Berg, 1983; Moon *et al.*, 1992), or consonant-vowel syllables (Bertoncini *et al.*, 1987; Moon *et al.*, 1992; Dehaene-Lambertz & Pena, 2001), although these segmental components of speech are not well represented in intrauterine recordings (Gri ths *et al.*, 1994).

Information contained in higher acoustic frequencies has been suggested to be essential for discriminating speech from its sine-wave analogs (SWA) by Vouloumanos and Werker (2007b). Frequencies above 500 Hz are severely attenuated, if available at all in utero. However, newborns are able to discriminate between speech sounds and its SWA only if higher frequencies are preserved. The neonates did not discriminate between speech and sine-wave stimuli when they were both low-pass filtered in order to emulate what fetuses are likely to hear inside the womb. With low-pass-filtered stimuli the sucking rate decreased when speech and SWA were alternating, indicating that they were perceived as being similar. Thus, the authors suggested the hypothesis that prenatal learning is implausible and raised the intriguing question of whether infants' preference for speech may be innate. However, given the prominence of the period of heightened learning ability shortly after birth, we cannot rule out that important environmental cues associated with human communication may be acquired with lightning speed within hours after birth.

Young infants can discriminate not only their native language phonetic contrasts but also phonetic contrasts of foreign languages which for adults sound indistinguishable (such as Hindi phonemes /Ta/-/ta/ for English-speaking adults [Werker & Tees, 1984a] or /r/ and /l/ for Japanese adults, which are combined in a single phonemic category in Japanese) (Kuhl *et al.*, 2006). It appears that a newborn's speech perception systems are initially broadly tuned and only further in development over the first few months of life do infants become specialized for the particular language they hear every day. This leads to a decline in non-native phonetic perception between 6 and 12 months of age. Several behavioral and electrophysiological studies support this view of perceptual narrowing and specialization. For example, in a study by Werker and Tees (1984b) only a few of the 10- to 12-month-old infants were able to discriminate non-English contrasts, whereas at the age of 6–8 months almost all infants could do it. Electrophysiological studies have also shown that discriminatory ERP responses are present for both native and non-native contrasts at about 6–7 months of age, whereas only for native contrast in 11- to-12-month olds (Cheour *et al.*, 1998; Rivera-Gaxiola *et al.*, 2005). At the same time, infants' responsiveness to native contrasts increases (Rivera-Gaxiola *et al.*, 2005).

What mechanism is responsible for this perceptual narrowing? Electrophysiological studies suggest that language specialization probably involves commitment of the brain's neural networks to native-language phonetic properties, a process that occurs within the first few months of birth (for a review, see Kuhl, 2004).

One hypothesis is that infants' brains use a stochastic learning mechanism for advancing in their native language. That is, their brains implicitly analyze the statistical distributions of sounds they hear in their environment. The distributional frequencies are low at the borders between categories. Infants' learning of distributional patterns appears to be very rapid; Maye and colleagues (2002) have shown that after only two minutes of exposure to phonetic stimuli with different distributional frequencies, infants showed differential patterns of response in an auditory preference procedure. Infants were familiarized with phonetic continuum between "da" and "ta" syllables in one of two distributional patterns. One group was presented with the "bimodal" frequency distribution, i.e., distributional frequencies at the endpoints of the continuum were higher than that at the central part. The second "unimodal" group heard more frequent presentations of stimuli from the middle of the continuum. The results demonstrated that infants are able to use distributional information to detect the phonetic category structure; infants from the "bimodal" group could discriminate the syllables at the endpoints of the continuum whereas infants from the "unimodal" group could not.

Understanding speech also requires knowledge, i.e., which strings of sounds are words and which are not. How do infants learn to understand where the one word ends and the next one begins? This learning may also be advanced by infants' sensitivity to statistical information derived from distributions of patterns of sound. Statistical patterns serve as cue to word boundaries, since the transitional probabilities between syllables are highest when the two sounds follow one another within a word, whereas transitional probability on the word boundary is relatively low.

In a study by Saffran and colleagues (1996), infants listened to a meaningless string of syllables presented continuously without any pauses, stress differences or intonational contours, containing four three-syllable pseudo-words. The only cues for "word" boundaries were transitional probabilities between the syllables; within pseudo-words the probability was 1.0, with all other adjacent syllables it was 0.33. In the experimental session, infants were presented either with "words," a familiar order of syllables, or "nonwords," which contained the same syllables that infants heard during familiarization but in a different order. Infants preferred to listen to the unfamiliar order of syllables (nonwords), demonstrating novelty preference. This study provided evidence that the infant brain uses some kind of distributional analysis for extracting regular patterns in speech that will give them access to linguistic structure.

The ability to extract the regularities in auditory input was recently documented to be present within three days of birth. In this study, newborns were shown to extract an abstract rule from the auditory sequence – the direction of a frequency change in pairs of tones (Carral *et al.*, 2005). The auditory sequence contained 87.5% tone pairs of ascending frequency (the second tone higher than the first) and 12.5% of pairs of descending frequency (the second tone lower in frequency than the first). The pairs were composed of seven frequency levels and six different physical pairs for each stimulus type, and the frequent and infrequent pairs were composed of the same frequencies, only in reversed order. Infrequent "deviant" pairs elicited a positive mismatch response in newborn's ERPs, indicating automatic detection in the brain of violation of the rule "the second tone higher than the first." This "abstract rule" extraction may be particularly important for language acquisition, since learning language requires extracting multiple rules of phonology, morphology, syntax, and semantics.

Electrophysiological studies have also shown that newborns are capable of very rapid auditory learning. In studies by Ruusuvirta and colleagues (2003) it has been shown that newborns implicitly "learn" to conjoin arbitrary auditory features (such as frequency, intensity, and duration), demonstrating a skill termed "auditory feature binding." The results have shown that a newborn's brain responds differentially to high- and low-probable combinations of auditory features, thus indicating that neural representation for the high-probable combination has been formed over repeated presentations. Newborns were shown to learn to discriminate acoustically very close vowels, after being exposed to repeated presentation of those while being asleep (Cheour *et al.*, 2002). The significant difference between responses before and after nocturnal training suggests that infants are better able to discriminate speech sounds after training, thus assimilating auditory information even while they are sleeping.

Within the first week of life infants are able to discriminate phonemes in a categorical manner, generalizing across different speakers (Dehaene-Lambertz & Pena, 2001). Dehaene-Lambertz and Pena (2001) compared brain response to phoneme change in neonates when irrelevant speaker variation

was present or absent. Two syllables /pa/ and /ta/ were pronounced by four female speakers, with syllable intonation and duration varying from speaker to speaker. Evoked brain responses to phonetic change were collected in the "same speaker" condition and in the "different speakers" condition. A mismatch response was recorded at the same latency in both cases suggesting that the phonemes were processed not merely on the basis of their acoustical properties but as belonging to categories.

Given that in our natural environment there are often several people speaking at once, it is also essential to assign strings of ever-changing speech sounds to the sources they belong to in order to be able to correctly extract linguistic patterns. To analyze multiple sources of speech, one needs to be able to separate concurrent sequences of sound, the process called "sound stream segregation." This process relies on temporal behavior of various acoustic parameters, of which spectral pitch is the most effective (Bregman, 1990). In adults, fast presentation of sounds from two different frequency ranges results in perception of two different sound streams, one of lower and one of higher frequency (Bregman, 1990). Recently it has been shown that newborn infants can also segregate the mixture of the concurrent sound streams from each other, and thus organize their auditory input according to the sound sources (Winkler *et al.*, 2003) (see Fig. 12.2 in color plate section). In a study by Winkler and colleagues (2003), in one condition (one-stream condition) the intervening tones were very close in frequency to standard and deviant tones of the oddball condition, thus integrating in a single sound stream and interfering with the detection of the repetition of the standard tone. In this condition, the brain responses to standard and deviant tones do not differ. In the other condition (two-stream condition), the tone frequencies of the intervening tones were lowered, thus being perceived as a separate stream, and then the deviant tones were processed differently from standard tones just as they were in the control condition (with no intervening tones). This ability underlies infants' orientation in the world and probably enables the separation of concurrent streams of speech.

In summary, newborns exhibit amazing speech perception capacities. Within the first days of their lives they extract regularities and bind auditory features together, they discriminate between languages, generalize across speakers, and organize auditory input according to the existing source.

Conclusions

In this review we have highlighted several aspects of newborn perception and behavior. We conclude that the traditional view of the human newborn as a passive reflex responder to external stimuli can no longer be supported by current evidence. Instead, the human newborn is equipped with a number of different powerful learning mechanisms in the olfactory, visual, and auditory domains. These learning mechanisms are guided and constrained by initial biases. Some of these biases are based on prenatal learning, particularly those in the olfactory and auditory domains, while others may depend on primitive subcortical routes for orienting to visual stimuli. New techniques such as optical imaging (near-infrared spectroscopy) and eye tracking will allow rapid development in the scientific understanding on the newborn's perceptual and cognitive world. There is no doubt that these studies will contribute to pivotal debates in developmental psychology and cognitive neuroscience about the role of postnatal experience in developing adult brain structure and function.

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Section 3
Chapter
13

Radiological and neurophysiological investigations

Imaging the neonatal brain

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Introduction

Ultrasound is the routine imaging tool for assessing and monitoring the neonatal brain. It is cheap, mobile, and easy to use, and in experienced hands serial examination provides valuable information about the normal and abnormal brain. Computed tomography exposes the already sick neonate to unacceptable radiation and may miss clinically important pathology. Magnetic resonance imaging (MRI) has become an invaluable adjunct to ultrasound: the two techniques have complementary roles with ultrasound providing an ideal screening tool. MRI is safe and can therefore be used serially; in addition, unlike ultrasound, it may be used when the anterior fontanelle has closed. Unfortunately MRI of the sick or very preterm neonate is not easy and experience is limited to relatively few centers, both in the practicalities of performing a successful examination and in the interpretation of the results.

The increasing literature on advanced postacquisition analysis of MR images is evidence of the power of this tool to investigate the normal and abnormal developing brain but these techniques depend on high-quality imaging datasets, which may be difficult to acquire. The aim of any neonatal MR examination, whether performed for clinical or research studies, is to produce high-quality datasets, safely and in an acceptable acquisition time.

Practical issues

Sedation

Successful imaging of the neonatal brain requires careful preparation of the infant and close cooperation between radiologist, radiographer, and neonatologist. Neonates may be successfully imaged during natural sleep, following a feed, or under light sedation (Rutherford, 2002). It is possible to successfully image neonates without sedation using air-evacuated bags and/or swaddling but this reduces the likelihood of obtaining a full examination. Images may be contaminated by motion that may not hinder visual analysis but makes objective quantification for research purposes impossible. Unsettled or unsedated neonates can be successfully imaged using fast brain sequences, at the expense of good signal-to-noise, or using a new approach recently developed for imaging the fetal brain using dynamic acquisition (Jiang *et al.*, 2007). Neonates can be sedated with chloral hydrate at a dose of 25–50 mg/kg administered orally, via a nasogastric tube, or rectally, approximately 15 minutes before the start of the examination. The infant should be nil by mouth for at least an hour prior to administration as this aids absorption of the chloral hydrate. Sedated neonates will usually sleep through a 45-minute examination. Severely encephalopathic neonates may not require sedation or may already be sedated by anticonvulsant medication. All infants, sedated or not, should be monitored during scanning with MR-compatible pulse oximetry and electrocardiography (ECG). A pediatric qualified member of staff should be in attendance throughout the scan.

Safety

Excessive noise, particularly with fast sequences such as diffusion-weighted or perfusion-weighted imaging, may wake a sleeping infant or potentially harm the developing auditory system, and ear protection should be used. We use moldable dental putty as individualized earplugs and neonatal earmuffs (Natus Medical, San Carlos, CA, USA). Infants may move even when asleep: molded air bags or foam placed snugly around the infant's head will keep this to a minimum. This combination reduces noise levels by approximately 35 dB. Swaddling the infants will keep them warm and also

The Newborn Brain: Neuroscience and Clinical Applications, 2nd edn., eds. Hugo Lagercrantz, M. A. Hanson, Laura R. Ment, and Donald M. Peebles. Published by Cambridge University Press.

Table 13.1 Suitable sequence parameters for examination of the neonatal brain

Sequence	TE (ms)	TR (ms)	TI (ms)	Slice thickness (mm)	Number of signals averaged	Matrix	Field of view (mm)	Other
T1-weighted conventional spin echo	15	500	–	4	2	192 × 256	220–240	
T2-weighted fast spin echo	208	4000	–	4	2	192 × 256	220–240	
T1-weighted volume acquisition	4.5	30	–	1.6	1	192 × 256	220–240	
Inversion recovery	30	3500	1000	4–5	2	192 × 256	220–240	
Diffusion tensor imaging	6000		90	4	1	112 × 112	240	*b* value, 750

reduce movements. A full metal check needs to be carried out with particular attention, in this population, to the presence of intravenous scalp lines, long lines, electroencephalography (EEG) electrodes, intraventricular shunts, patent ductus arteriosus (PDA) clips, and metal fasteners on baby clothes. All staff involved in neonatal imaging need to be trained in MR safety. Full resuscitation equipment should be available immediately outside the scanner room.

Hardware and software adaptations

Good-image quality requires a high signal-to-noise ratio (SNR): this can be maximized by using a closely fitting coil. In the absence of a dedicated neonatal head coil, an adult knee coil will usually accommodate an infant up to six weeks post term. Phased-array coils provide improved benefit in terms of SNR even if designed for the adult brain. In the absence of MR-compatible ventilator equipment neonates can be safely hand bagged during a short MR examination. A larger adult-type coil may be necessary to accommodate the endotracheal tube in ventilated neonates. The most frequent reasons for poor-quality imaging are motion artifact and poor SNR.

The majority of neonatal studies have been performed at 1 Tesla (T) or 1.5 T but 3 T scanners are now commercially available and may eventually replace many 1.5 T systems, particularly for brain imaging. Conventional MR sequences are referred to as T1-weighted or T2-weighted. T1 and T2 refer to the MR parameters longitudinal (T1) and transverse (T2) relaxation times. Most MR sequences designed for imaging the adult brain need to be adapted to obtain good-quality images of the immature brain with its higher water content. The exact imaging parameters depend on the specific system and magnet strength being used. Representative parameters for conventional sequences in neonatal brain imaging at 1.5 T are shown in Table 13.1.

A neonatal brain examination should include the following sequences:

- T1-weighted sequence acquired in the transverse plane: this is ideal for assessing the basal ganglia and thalami (BGT) and provides the best view of the posterior limb of the internal capsule (PLIC) (Fig. 13.1).
- T2-weighted sequence acquired in the transverse plane: this is better than T1-weighted imaging for identifying early ischemic change (Fig. 13.2) and provides excellent detail of the developing white matter in the preterm infant (Fig. 13.3).
- T1-weighted sequence acquired in the sagittal plane: this provides the best views of the cerebellar vermis, and an excellent view of the brainstem, the sagittal sinus, and the corpus callosum. It is also good for identifying extracerebral hemorrhage.
- Volume acquisition: this is ideal as it provides thin slices, can be reformatted into any plane and can be used for absolute quantification of brain structures with manual or automatic segmentation techniques.
- Diffusion-weighted imaging (DWI): this is ideal for early (<1 week) identification of ischemic parenchymal tissue (Fig. 13.2). Results for white matter are, however, more consistent than for BGT injury. Diffusion tensor imaging with multiple planes of sensitization is an invaluable research tool for studying brain tissue microstructure.

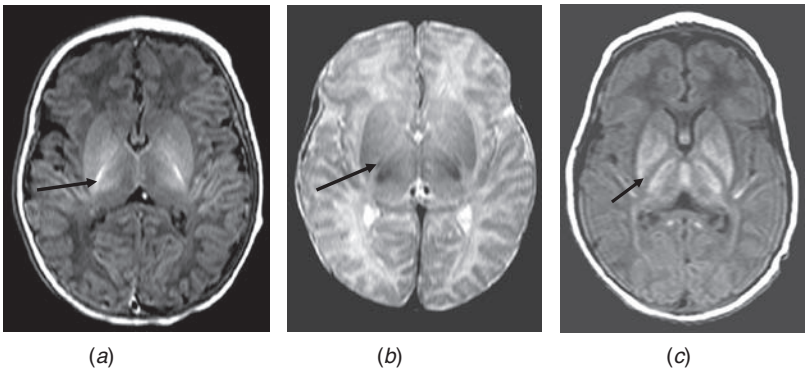


Fig. 13.1 The posterior limb of the internal capsule (PLIC). (*a*) T1-weighted and (*b*) T2-weighted images in the transverse plane. Normal appearances of the neonatal brain in a term-born neonate. There is evidence of myelination within the PLIC seen as high signal intensity in (*a*) and less obvious low signal intensity in (*b*) (arrows). (*c*) T1-weighted image: abnormal appearances in a term-born neonate with severe basal ganglia and thalamic injury. There is no high signal from myelin within the PLIC, which has a uniform abnormal low signal intensity (arrow).

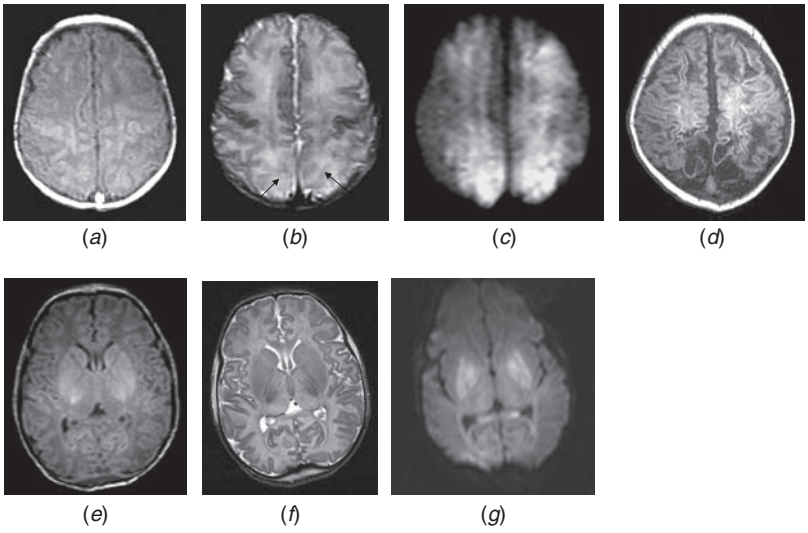


Fig. 13.2 Diffusion-weighted imaging ([DWI] Rutherford, 2002).

White matter infarction: term-born neonate aged 3 days. (*a*) T1-weighted image; (*b*) T2-weighted image; (*c*) DW image. Level of the centrum semiovale in the transverse plane. There is a loss of gray/white matter differentiation, which is most obvious on the T2-weighted image (*b*). The areas of abnormality are very obvious on the DW image (*c*) where the acute infarction has an abnormally high signal intensity. (*d*) Established infarction in the same infant aged 3 weeks. T1-weighted image showing cystic breakdown in areas that showed loss of tissue differentiation on conventional imaging and abnormal high signal on DWI (Jiang *et al*., 2007).

Basal ganglia and thalamic injury (BGT). (*e*) T1-weighted, (*f*) T2-weighted, and (*g*) DW image. There are abnormal high signal intensities within the BGT on the T1-weighted image (*e*) and a combination of high and low signal intensities on the T2-weighted image. (*f*) There is abnormal high signal intensity but mainly in the lentiform nuclei on the DW image (*g*).

In addition, the following may be added: a venogram to exclude the presence of sinus thrombosis and differentiate this from subdural hemorrhage; and MR angiography, to look at both cerebral and neck vessels, which may be abnormal in focal stroke. Intravenous contrast, gadolinium dimeglumine gadopentetate at a dose of 0.2 ml/kg, may be used in suspected infection, e.g., herpes encephalitis or cerebral abscess. We do not routinely obtain a fluid-attenuated inversion recovery (FLAIR) image in infants, but this sequence will, however, often accentuate abnormalities within the white matter. After 1 year of age FLAIR imaging may be used, as for adults, to detect areas of abnormal gliosis. This is useful in periventricular leukomalacia (PVL), where the glial tissue may be difficult to differentiate from cerebrospinal fluid (CSF) on T2-weighted images (Fig. 13.4).

The normally developing brain

As the role for MRI continues to expand, the need for an appreciation of the normal appearances for each MR sequence in each plane and at each gestational age

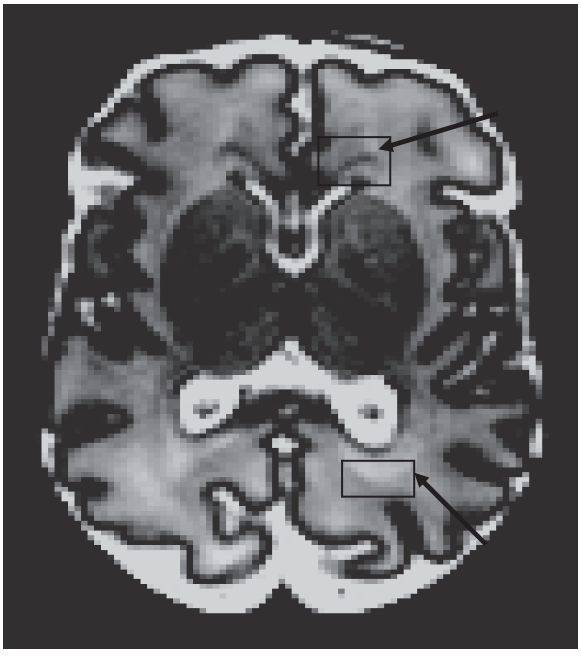
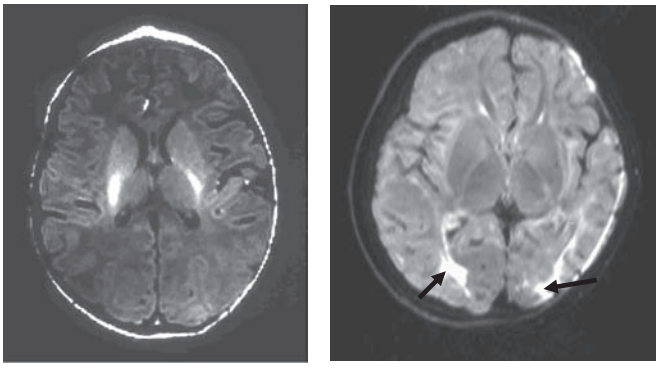


Fig. 13.3 Fast T2-weighted images of a preterm infant imaged at 32 weeks postmenstrual age. There are foci of high and low signal intensity in the developing white matter seen as caps (anteriorly) and arrowheads (posteriorly). These alternating signal intensities are thought to correspond to resident microglial cells (low signal intensity) and developing white matter tracts (high signal intensity) or crossroads (Judas *et al.*, 2005).

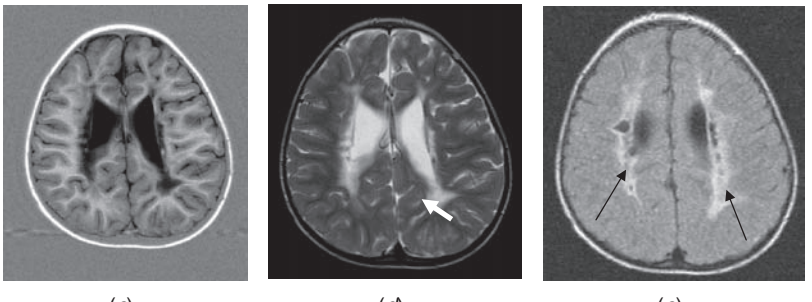
becomes essential. It is possible to image normal term-born neonates (Fig. 13.1) but large numbers are needed to ensure the full range of "normal" appearances can be documented. In addition, all data obtained from apparently normal neonates need to be combined with long-term neurodevelopment information about the child. The normal term neonate provides only one time-point in the development of the brain and scanning after the neonatal period becomes increasingly difficult, as sedation of the normal infant is not usually approved. Most post-term studies rely on infants being scanned for reasons other than the central nervous system (CNS) or assume that in the absence of obvious pathology a scan performed for perhaps isolated seizures can be taken as representing the normal.

Fetal MR imaging

Modern fast, single-shot, T2-weighted MRI allows us to image the mobile fetus and hence obtain information on the immature brain from approximately 18 weeks' gestation. The accumulation, careful review, and objective analysis of normal fetal data provide unprecedented in vivo information on normal brain growth and development (Fig. 13.5). Fetal motion



(*a*) (*b*)



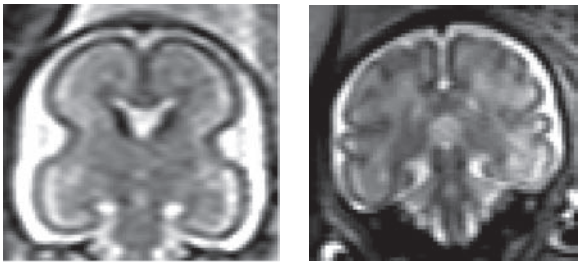
(*c*) (*d*) (*e*)

Fig. 13.4 Parasagittal infarction in a term-born neonate. (*a*) T1-weighted image at five days showing loss of gray/white matter differentiation. (*b*) Fluid-attenuated inversion recovery (FLAIR) image at age 1 year showing white matter atrophy and abnormal high signal intensity (arrows) consistent with glial tissue. (*c*) Imaging of an infant with cerebral palsy showing abnormalities consistent with periventricular leukomalacia. Abnormal high signal intensity (arrow) from glial tissue within the periventricular white matter in (*d*) is more easily differentiated from cerebrospinal fluid and persisting periventricular cysts in the FLAIR image (*e*) (arrows).

limits image acquisition to single-shot techniques and quantification, therefore to two-dimensional measures of the brain and its structures. Recently a new approach to fetal motion using a dynamic acquisition of single-shot, T2-weighted data to oversample the brain has been developed (Jiang *et al.*, 2007). Following acquisition of the raw single-shot images these can be registered to produce high signal-to-noise high-resolution volumetric datasets (Fig. 13.6). These datasets can then be quantified manually or with automatic techniques to provide novel in vivo information about normal brain growth and development. This information could be compared to gestationally age-matched preterm datasets to establish at what stage the brain growth and development in the preterm infant starts to deviate from normal.

Conventional MR sequences

Most units restrict their imaging examination to conventional T1- and T2-weighted MR sequences. If these sequences have been adapted for the higher water content of the immature brain, have been acquired in appropriate planes, and the images are free of artifacts they will provide a wealth of useful data.



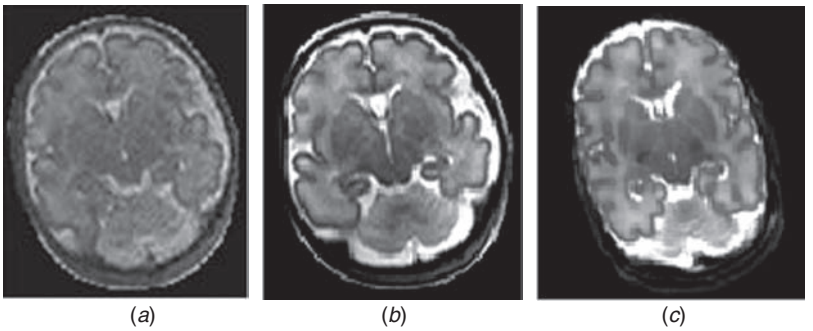
(*a*) (*b*)

Fig. 13.5 T2-weighted images in the coronal plane of the normal fetal brain at (*a*) 20 weeks' gestation and (*b*) 34 weeks' gestation showing an increase in cortical folding. The high signal intensity medial to the low signal intensity cortex represents the transient subplate. In the more mature folded cortex the subplate is only visible on the top of gyrae (*b*).

The term-born infant

In the term-born infant conventional imaging will confirm the presence of normal anatomy, exclude congenital malformations, show evidence of established antenatally acquired injury, and identify perinatal injury. In infants with perinatal injury the pattern of lesions on T1- and T2-weighted images may be used to predict neurodevelopmental outcome. For this purpose it is best to scan between one and four weeks from delivery, when the lesions have evolved and are at their most visible, but before tissue has started atrophying making it more difficult to ascertain the exact nature of the original injury (Fig. 13.7).

In infants with a history of hypoxic–ischemic encephalopathy (HIE) it is now well established that the appearance of the PLIC is an excellent predictor for motor outcome. (Rutherford *et al.*, 2004; Cowan & de Vries, 2005) (Fig. 13.1). The severity of associated BGT lesions determines the severity of motor outcome (Fig. 13.8) with severe lesions being associated with white matter atrophy and secondary microcephaly and additional severe cognitive impairment (Rutherford, 2002; Okereafor *et al.*, 2008). Isolated white matter injury in the encephalopathic term-born infant is associated with cognitive impairments but motor function is usually normal unless the white matter injury is extensive (Fig. 13.9) (Cowan *et al.*, 2003). In term-born infants with focal injury, such as middle cerebral artery infarction, abnormal motor outcome in the form of a hemiplegia is associated with three-site involvement, the parenchymal white matter, BGT, and the PLIC (Mercuri *et al.*, 1999) (Fig. 13.9). Infants with symptomatic hypoglycemia show white matter injury although this is not always restricted to



(*a*) (*b*) (*c*)

Fig. 13.6 Fetus of 33 weeks' gestation. Single-shot, T2-weighted image dynamically acquired at 1.5 T in the (*a*) coronal plane post acquisition and (*b*) post registration by computer program of four loops of data (Jiang *et al.*, 2007). The registered image shows high signal-to-noise and high resolution comparing favorably with a postnatal image acquired at 3 T in an infant of similar postmenstrual age (*c*).

the posterior parietal lobes as previously reported (Burns *et al.*, 2008) (Fig. 13.8). Once again cognitive impairment is common with moderate and severe white matter injuries. Neonates with hyperbilirubinemia who develop kernicterus may show abnormal signal intensity within the globus pallidus on neonatal scans. However, abnormal signal intensity may not be obvious until a few months of age, appearing as high signal intensity on T2-weighted images and, to a lesser extent, as low signal intensity on T1-weighted images. A normally appearing neonatal MR scan, therefore, in an infant with marked hyperbilirubinemia does not predict a normal outcome.

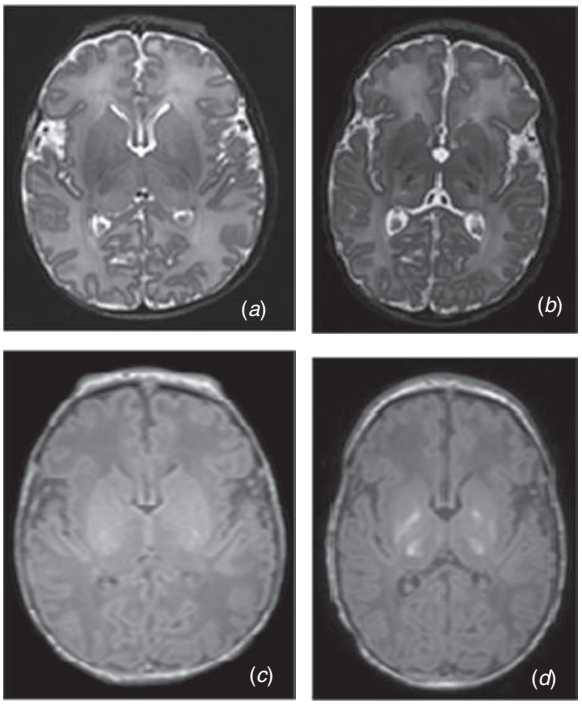


Fig. 13.7 Evolution of basal ganglia and thalami (BGT) lesions in a term-born infant with hypoxic–ischemic encephalopathy (HIE). Images at (*a*, *c*) four and (*b*, *d*) 18 days: (*a*, *b*) T2- and (*c*, *d*) T1-weighted sequences. Appearances of the BGT at four days are not normal. There is a reduced signal intensity from myelin in the posterior limb of the internal capsule (PLIC) in (*c*) whereas in (*a*) the low signal intensity from myelin is excessive. The abnormal signal intensities have evolved so that at 18 days of age the changes are very obvious. In addition, on the later scans (*b*, *d*) in both sequences the signal intensity within the PLIC is absent.

The preterm infant

The MR appearances of the optimal preterm at different gestational ages are well documented (Maalouf *et al.*, 1999; Counsell *et al.*, 2002; Rutherford, 2002; Dyet *et al.*, 2006), although imaging prior to term has only been performed in a few centers. Early brain MRI in the preterm infant allows an earlier description and better definition of recognized acquired lesions such as PVL, or hemorrhagic venous infarction (Fig. 13.10). MRI has also identified new white matter disorders in the preterm infant, which were not previously recognized from ultrasound studies, e.g., punctate white matter lesions. (Childs *et al.*, 2001; Ramenghi *et al.*, 2007). Many optimal preterm infants without overt focal brain lesions still show visual abnormalities on conventional MRI sequences at term-equivalent age. These include mild ventricular dilation in the absence of germinal matrix/intraventricular hemorrhage, a widened extracerebral space, decreased cortical folding and altered signal intensity within the white matter. The latter has been termed DEHSI – diffuse excessive signal intensity because of the marked high signal intensity on T2-weighted images (Fig. 13.10). (Maalouf *et al.*, 1999; Dyet *et al.*, 2006).

These abnormal appearances at term-equivalent age have now been more objectively quantified in studies using both conventional sequences and diffusion tensor imaging (Ajayi-Obe *et al.*, 2000; Counsell

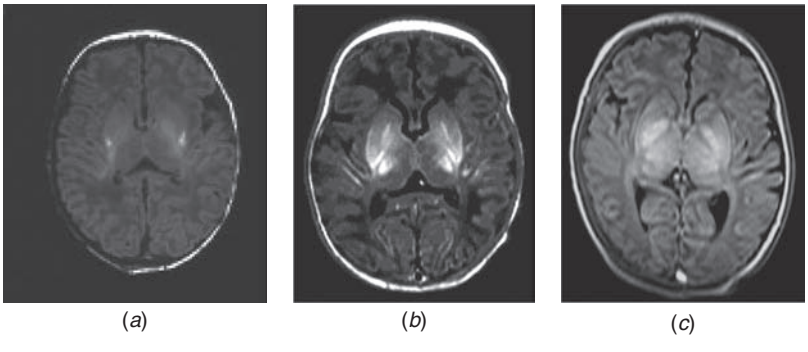
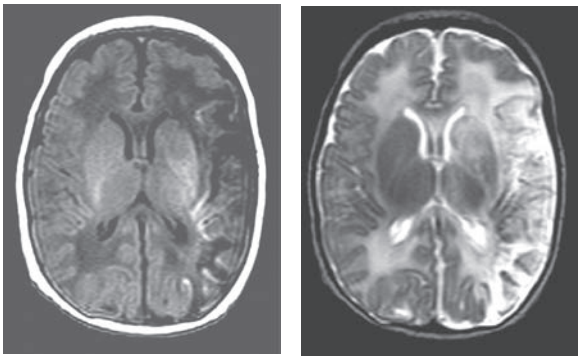


Fig. 13.8 T1-weighted images in three different term-born infants with hypoxic–ischemic encephalopathy showing (*a*) mild, (*b*) moderate, and (*c*) severe abnormal signal intensity within the basal ganglia and thalami.

et al., 2006; Kapellou *et al.*, 2006). The clinical implication of these early findings is now being reported as these children approach school age (Dyet *et al.*, 2006). The aim of many of the MR studies performed at term-equivalent age is to identify the neuroanatomical correlates for neurocognitive impairments in children who were born preterm. Imaging at term-equivalent age also allows prediction of motor outcome in infants with acquired lesions, with visual abnormalities in the myelination of the PLIC being associated with motor impairment in infants with focal unilateral lesions and with a lack of independent walking in PVL (Cowan & de Vries, 2005).



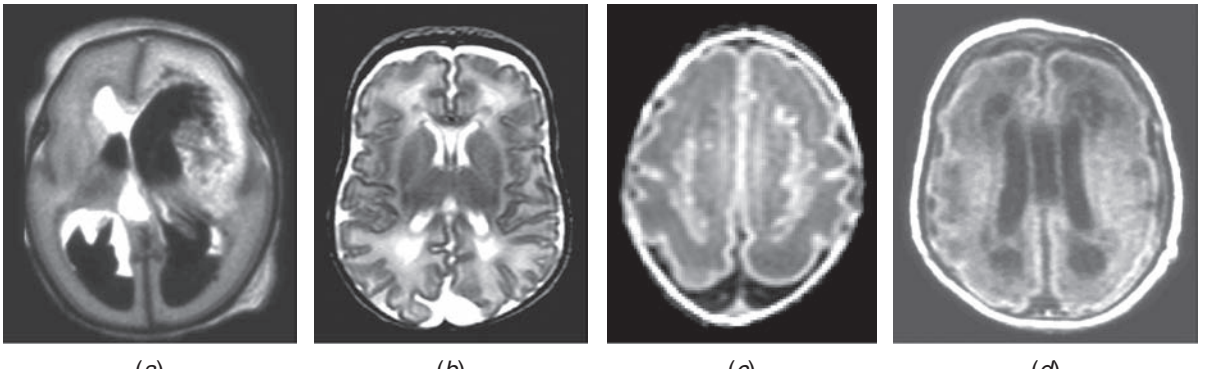
(*a*) (*b*)

Fig. 13.9 Perinatal left-sided middle cerebral artery infarction in a term-born infant: (*a*) T1- and (*b*) T2-weighted images. There is abnormal signal intensity within the basal ganglia, thalami, and posterior limb of the internal capsule, in addition to the parenchymal white matter and cortex. These changes would be associated with the later development of a right-sided hemiplegia.

Advanced imaging techniques

Diffusion tensor imaging

DWI uses sensitizing gradients in three orthogonal planes to produce images that are "weighted" by the random or Brownian motion of water within the brain tissue. In brain tissue this motion is not random but is restricted by barriers, such as cell membranes, and therefore shows directionality. To measure this directionality at least six sensitizing gradients must be applied, a technique known as diffusion tensor imaging. Directionality or anisotropy is present in white matter tracts where diffusivity is greatest in a direction parallel to the tracts but is relatively restricted across them. Anisotropy increases with myelination but does exist, although to a lesser degree, in earlier development as a result of normal cell membranes and premyelination changes around the axons. The role of DWI in clinical imaging is undisputed with overt visual changes, consistent with restricted diffusion, occurring in the presence of very acute ischemia, when the appearances of conventional imaging may be considered normal (Fig. 13.2). These visual changes are due to a reduction in free water motion or diffusivity as a consequence of acute cellular swelling and imminent necrosis. In the neonatal brain this may arise as a consequence of global hypoxic–ischemic injury such as in HIE and in focal stroke or injury secondary to hypoglycemia. Diffusivity can be quantified as an apparent diffusion coefficient (ADC), levels of <1 in neonatal white matter being associated with irreversible infarction (Rutherford *et al.*, 2004). Similar changes in the BGT are less



(*a*) (*b*) (*c*) (*d*)

Fig. 13.10 White matter lesions in the preterm infant. (*a*, *b*) T2- and (*c*, *d*) T1-weighted images. (*a*) Bilateral intraventricular hemorrhage with a large left-sided hemorrhagic venous infarction. (*b*) Diffuse excessive high signal intensity in the white matter of a preterm infant at term-equivalent age. (*c*) Severe bilateral punctate lesions in the white matter of the centrum semiovale. (*d*) Bilateral periventricular cysts in a preterm infant with periventricular leukomalacia.

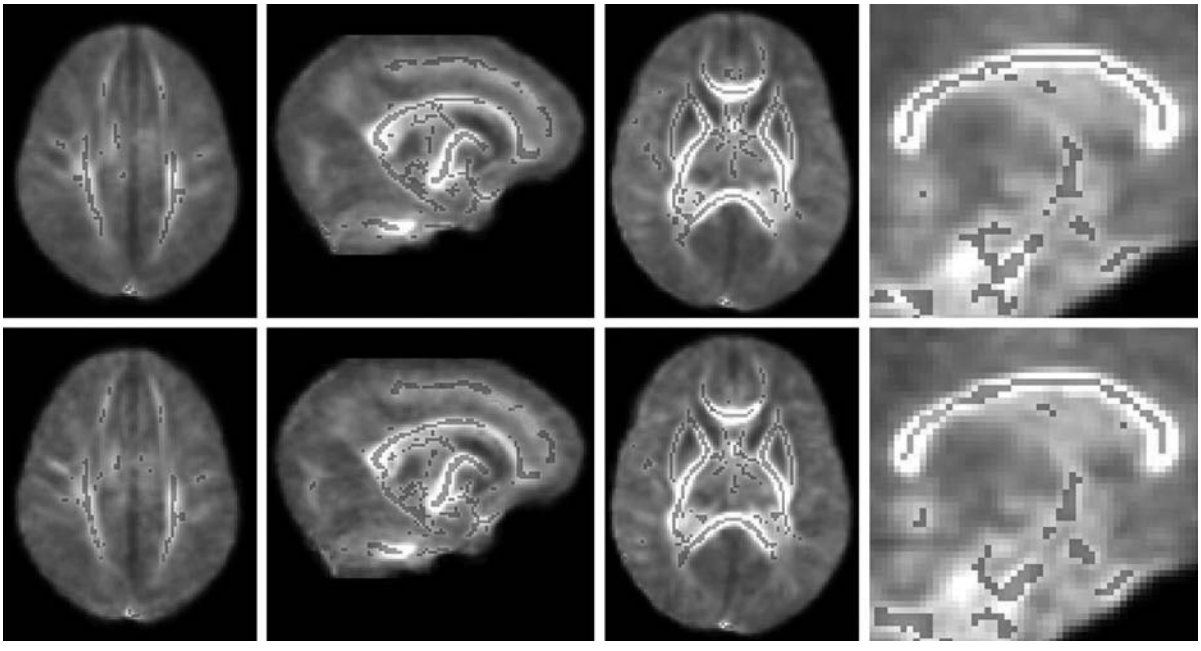


Fig. 13.11 (See color plate section.) Tract-based spatial statistics (TBSS). Using diffusion tensor imaging, an atlas of white matter tracts is derived from values of fractional anisotropy (FA). The top row compares the FA between 26 preterm infants at term-equivalent age against six term-born controls. Regions in green showed no difference in FA between the groups, whereas regions in blue showed a significantly lower FA in the preterm group, correcting for multiple comparisons using cluster-based thresholding. The bottom row shows the comparison between 11 preterm infants at term that were born ≤28 weeks' gestation compared with the six term controls. Regions in blue indicate a significantly lower FA in the preterm group (Anjari *et al.*, 2007).

predictable where the presence of heterogeneous abnormalities may reflect different forms of cell death or injury in gray matter. ADC values in injured white matter pseudonormalize around one week from the onset of ischemia, when cell membrane breakdown allows free motion of water. Fortunately, conventional imaging is visually abnormal at this stage. Measures of the direction or fractional anisotropy (FA) of the water motion can also be quantified and will not pseudonormalize following overt infarction as directionality of the tissue will have been permanently disrupted (Ward *et al.*, 2006).

Diffusion imaging may also be used to study tissue microstructure in the developing brain and has become a valuable technique for identifying more subtle abnormalities in the tissue microstructure. In the preterm infant imaged at term-equivalent age, the subjective visual appearance of DEHSI has been further investigated with diffusion tensor imaging associated with abnormal diffusion characteristics. Regional analysis of white matter shows that DEHSI has higher ADC and lower FA (Counsell *et al.*, 2006); in addition, in the preterm infant, the ADC of a large area of white matter within the centrum semiovale has been associated with poorer developmental quotients at 2 years of age (Krishnan *et al.*, 2007).

Diffusion tractography techniques allow white matter tracts to be mapped and has been used to show asymmetries in tract development in infants with focal lesions (Counsell *et al.*, 2007). This approach can also be used to establish a functional anatomical relationship and an association with visual function at term-equivalent age and FA values within the developing optic radiation has recently been shown (Bassi *et al.*, 2008). Tract-based spatial statistics (TBSS) allow a nonhypothesis-driven "screen" of the entire brain for values of FA that are associated with a specific variable. Using this technique, Anjari and colleagues (2007) have shown that abnormalities in the preterm infant at term-equivalent age are widespread (Fig. 13.11).

Volumetric analysis: segmentation and morphometry techniques

There are now many studies on manual or automatic segmentation of preterm and term datasets to establish alterations in brain development associated with

preterm birth. Thalamic volumes are reduced in preterm infants even in the absence of lesions (Boardman *et al.*, 2006; Srinivasan *et al.*, 2007). Cerebellar volumes may be reduced at term-equivalent age but only in the presence of supratentorial lesions (Srinivasan *et al.*, 2006). Reductions in cortical gray matter volume have been associated with PVL (Inder *et al.*, 1999). In a small group of preterm infants a semiautomatic method for assessing curvature of the cortex showed that folding was reduced in preterm infants at term-equivalent age compared with term-born controls (Ajayi-Obe *et al.*, 2000). This approach was also used to demonstrate a reduction in cortical folding in infants at term who were exposed to multiple doses of antenatal steroids (Modi *et al.*, 2001). The relationship between growth of surface area in comparison with whole brain volume has been explored using MR images of a large cohort of preterm infants (Modi *et al.*, 2001). This study showed that surface area growth was affected by decreasing gestational age at birth and male sex. Three-dimensional assessments of cortical curvature are now being explored. Automatic segmentation techniques (Thompson *et al.*, 2007) have been used to relate perinatal factors to regional cerebral volumes. Optimization of automatic methods for segmentation is still needed, as problems with partial volume effects, which are often underestimated, have been difficult to solve (Xue *et al.*, 2007a, b).

More recently, postacquisition techniques enabling examination of large numbers of datasets have been applied to cohorts of preterm infants. These include deformation-based morphometry which can identify statistically significant alterations in the volume of brain structures or tissue types (Boardman *et al.*, 2007; Aljabar *et al.*, 2008). Using this approach it has been shown that preterm infants have a reduced thalamic volume at term-equivalent age when compared with term-born controls (Boardman *et al.*, 2006). This reduction was greatest in those with white matter disease. The technique was also used to show that there were no significant differences in brain volume in preterm infants at term-equivalent age when compared with term-born controls (Boardman *et al.*, 2007).

Angiography

It is difficult to obtain good quality MR angiograms because of the small size of neonatal vessels and the relatively slow blood flow. The development of an optimized angiography technique at 3 T has shown that the preterm infant shows less tortuosity in the proximal cerebral vessels when compared with term-born controls (Fig. 13.12) (Malamateniou *et al.*, 2006). The reasons for this remain unclear.

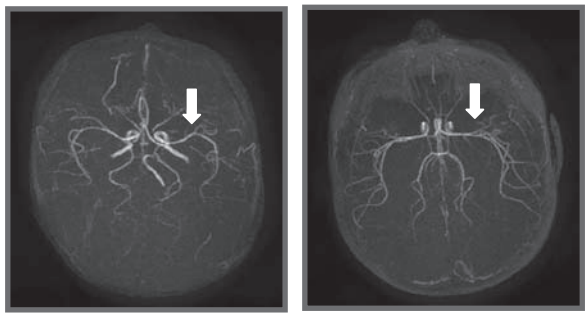


Fig. 13.12 Optimized MR angiography obtained at 3 T in (*a*) a term-born neonate and (*b*) a preterm infant at term-equivalent age. The middle cerebral artery appears more tortuous in the term-born neonate compared with the preterm neonate at term-equivalent age (arrows) (Malamateniou *et al.*, 2006).

Perfusion-weighted imaging

Perfusion-weighted imaging in the immature brain can be achieved by two techniques: contrast enhancement (Tanner *et al.*, 2003) and arterial spin labeling (ASL) (Miranda *et al.*, 2006). This does not require the injection of an exogenous contrast agent and is therefore more suitable for studying the neonatal brain; however, the resultant changes in signal intensity are very small. ASL still requires optimization and problems with quantification need to be addressed (Miranda *et al.*, 2006). Many recent models for perinatal brain injury stress the importance of infection and inflammation as causative factors but in the term infant and to a lesser degree in the preterm infant disorders of perfusion remain important factors. A noninvasive technique that could serially quantify perfusion in the very preterm or sick neonate may help refine clinical management.

Postmortem imaging

In the past few years attention has turned to the role of postmortem MRI. This has largely arisen as a consequence of the UK organ retention scandal and a subsequent unwillingness both of doctors and parents to consider conventional postmortem. In addition an increasing reluctance for postmortem exists in many countries for religious reasons. It is not yet clear how useful postmortem imaging is in the absence of

conventional autopsy, but it is able to identify abnormalities that may have been suspected or detected with ultrasound (Griffiths *et al.*, 2005). In certain circumstances postmortem imaging is better than autopsy, e.g., to establish the relationship of structures to each other or to confirm the presence of structures such as the corpus callosum or the position of the cerebellum. These features are often lost when the brain is removed from the skull at postmortem, particularly in the very preterm brain where pregnancy may have been terminated by intracardiac potassium.

Postmortem imaging in conjunction with conventional autopsy increases our ability to study the developing brain. Such combined studies also result in an improvement in our interpretation of MR images allowing us to establish histological correlates for MR appearances. This has been shown in elegant studies by Judas and colleagues (2005), which have established imaging correlates for resident microglia and so-called white matter crossroads. Current comparisons of imaging findings following clinical scans, performed either antemortem or postmortem, with histological comparisons involve only small series or case studies (Felderhoff-Mueser *et al.*, 1999; Jouvet *et al.*, 1999; Nicholl *et al.*, 2007).

MR imaging as a surrogate marker for outcome

The ability to predict outcome accurately from an early conventional MRI has been shown by studies on neonates presenting with encephalopathy or with isolated seizures. In the former the presence of abnormal signal in the PLIC predicts a motor impairment with a 0.9 sensitivity and 1.0 specificity (Rutherford *et al.*, 2004). Similarly in preterm infants with focal lesions an asymmetry of the appearances of the PLIC at term-equivalent age predicts motor impairment (De Vries *et al.*, 1999). The numerous studies to date in perinatal brain injury allow us to predict which infants are at the highest risk of neuromotor impairments and this information can be used to ascertain which infants might benefit from intervention. The presence of an abnormality on MRI may indicate that it is already too late for early interventions, e.g., hypothermia, but may be an entry criterion for future interventions designed to decrease secondary injury, prevent atrophy in connected structures and promote repair of injured axons or to assist in their attempts to reconnect following the death of their target neurons.

MRI is excellent at predicting outcome in preterm infants with parenchymal infarction or PVL. There are few studies using MRI to predict outcome in a large cohort of preterm neonates with isolated intraventricular hemorrhage, with or without surgical intervention. The majority of preterm infants do not have focal lesions but may show isolated DEHSI on term-equivalent MRI and this has been associated with poorer developmental outcome at 2 years of age but not with overt motor impairment (Dyet *et al.*, 2006).

Imaging correlates for later neurocognitive impairments are subtler and may be difficult to ascertain with the use of conventional MRI sequences and visual analysis. Ongoing studies comparing the results of advanced imaging techniques and postacquisition quantification with long-term outcomes will allow us to identify early markers for cognitive impairments. Lastly, MRI provides an ideal technique for monitoring the effects of neonatal interventions designed to reduce brain injury and promote normal brain growth and development as and when they become available (Rutherford *et al.*, 2005).

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Chapter 14

Electroencephalography and amplitude-integrated EEG

Lena Hellström-Westas and Ingmar Rosén

Introduction

The electroencephalogram (EEG) records electrical activity from the cerebral cortex. This activity is derived from synchronized postsynaptic action potentials from large numbers of neurons, re ecting the functional state of the brain. Thousands of publications have described the EEG during normal and abnormal conditions, in subjects of di erent ages, including very preterm newborn infants. Spontaneous recurrent action potentials within thalamocortical relay cells, the reticular thalamic nucleus, and cortical pyramidal cells constitute the basis for the EEG activity (Steriade *et al.*, 1990). This activity is synchronized by recurrent connections between the thalamocortical relay cells and the reticular thalamic nucleus, and by thalamocortical connections. In adults, intracortical connections generate higher frequency EEG components during mental processes and active wakefulness. During arousal, cholinergic (and norepinephrinergic) a erents from the brainstem exert an excitatory depolarizing e ect on thalamocortical and cortical cells and inhibit the reticular thalamic cells. The net result of arousal is a reduction of synchronous low-frequency activity, and an increase of asynchronous high-frequency activity. The neurophysiological basis for the EEG in newborn preterm and term infants is not very well known. The cortical subplate zone, a structure that is present in the fetus during the second trimester and which is the origin of thalamocortical and corticocortical a erents, probably modulates EEG activity via cortical connections (Kostovic & Jovanov-Milosevic, 2006). The subplate zone is probably also important for the mechanisms underlying spontaneous activity transients (SAT) in the discontinuous EEG of very preterm infants. The SATs are characterized by very-low-frequency waves with higher-frequency components superimposed. They correspond to intermittent periods with delta activity, as recorded by standard EEG and could be present as early as at 24 gestational weeks (Vanhatalo & Kaila, 2006). Experimental data indicate that the very-low-frequency EEG activity may be related to developmental brain wiring processes. Very-low-frequency activity has also been recorded in full-term infants (Thordstein *et al.*, 2005).

The recorded EEG activity is conventionally categorized into four main frequency bands: delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–13 Hz), and beta 13–30 Hz). The recorded EEG signal is very sensitive to external activity, and it is standard to use both high-pass filters (activity above a preset limit, often 0.3–0.5 Hz) and low-pass filters (usually activity below 70 Hz) to exclude extracerebral sources of activity such as movements, sweating, and electrical equipment. Electrocardiogram, respiration, and eye movements are usually recorded simultaneously to facilitate the interpretation of the EEG.

A standard EEG from a newborn infant is often recorded from 9–15 electrodes applied over the scalp according to the modified international 10–20 system. The number of electrodes can be varied according to the actual needs and to local traditions. During long-term EEG monitoring, fewer electrodes are used. On the other hand, in some research studies electrode caps have been used allowing recording from 64, 128, or more electrodes. Modern amplifier development, allowing high-input resistance, facilitates high spatial density recordings. The international 10–20 system describes electrode positions with reference to defined landmarks on the skull, which makes it possible to correlate EEG activity with anatomical landmarks. A capital letter denotes the position, e.g., F for frontal, C for central, T for temporal, O for occipital, followed by a number, with even numbers indicating the right side and odd numbers the left side.

The Newborn Brain: Neuroscience and Clinical Applications, 2nd edn., eds. Hugo Lagercrantz, M. A. Hanson, Laura R. Ment, and Donald M. Peebles. Published by Cambridge University Press.

Interpretation of EEG

Interpretation of the neonatal EEG requires experience and training. Visual inspection of the EEG and pattern recognition constitutes the basis for interpretation. This interpretation includes an evaluation of the dominating background activity and frequencies, topographic localization of activity, interhemispheric synchrony, and temporal development of the EEG including presence of sleep–wake cycling. The visual interpretation also includes evaluation of possible abnormal activity, such as abnormal background patterns or epileptiform activity. Most EEGs are recorded with digital technology, which makes it possible to perform quantitative analyses such as power spectral analysis, usually based on fast Fourier transformation (FFT).

The term "brain mapping" is sometimes used to describe the topographical distribution of EEG power. Analysis of EEG coherence is a method for investigating interactions between di erent parts of the cerebral cortex. This method was, for example, used to show increased cerebral maturation in preterm infants subjected to NIDCAP (Newborn Individualized Developmental Care and Assessment Program) care as compared with preterm controls (Als *et al.*, 2004). Spectral edge frequency (SEF) is a measure that can be obtained from power spectral analysis, and is calculated as the frequency below which a certain amount (often 80%–95%) of the power in the power spectrum resides. Power spectral analysis can be severely in uenced by artifacts, and this is a major reason why this method has not yet gained widespread use for clinical interpretation of EEGs in newborns.

In addition to electrical fields, the neuronal cortical current sources produce electromagnetic activity in the brain, which may be recorded by magnetoencephalography (MEG). MEG recordings are quite similar to EEG recordings performed in newborn infants at comparable maturation, and they can also be recorded from fetuses. Furthermore, MEG has been used in studies evaluating responses to fetal visual stimulation, auditory discrimination, and short-term memory functions (Eswaran *et al.*, 2002). Consequently, MEG has a large potential for investigating normal and abnormal fetal physiology. The method, however, is currently only available at a few centers worldwide.

Normal maturation of the EEG

EEG background patterns and conceptional age

The dominating EEG background pattern changes with the infant's conceptional age, and is also in uenced by sleep–wake states. Knowledge about maturational time cues in the electrocortical activity is essential for correct understanding of the neonatal EEG. The terms postconceptional/conceptional age (PCA) and postmenstrual age (PMA), which are summaries of the gestational age at which the infant was born plus the postnatal age, are used to describe the infant's maturation. A few studies including serial or continuous measurements of EEG background activity indicate that the amount of EEG activity increases during the first days of life in preterm infants (Greisen *et al.*, 1987; Victor *et al.*, 2005a; West *et al.*, 2006a). It is not known if this development is related to the birth process.

The development of the normal EEG maturation in preterm and term infants is well known (Tharp *et al.*, 1989; Lamblin *et al.*, 1999; Mizrahi *et al.*, 2004). An increasing number of studies are also published on the normal EEG of the extremely low gestational age infant, some of them also including long-term follow-up (Hayakawa *et al.*, 2001; Vecchierini *et al.*, 2003).

The overall EEG background can be classified as being mainly continuous or discontinuous. Full-term infants have continuous activity during wakefulness and active sleep, but during quiet sleep they can have discontinuous activity (*tracé alternant* pattern) or high-voltage slow activity (HVS). Continuous low-voltage pattern is an abnormal background pattern with an undi erentiated and very-low-amplitude EEG. In asphyxiated full-term infants this EEG pattern is associated with adverse outcome.

The discontinuous background of the preterm infant (*tracé discontinu*), and the *tracé alternant* pattern during quiet sleep in term infants are normal discontinuous background patterns. Burst suppression di ers from *tracé discontinu* in many aspects – it is unresponsive and usually synchronous – and the activity during the interburst interval (IBI) is of very low voltage or entirely absent. Burst suppression is an abnormal pattern that may be seen in association with brain injury or deep sedation. An inactive or

isoelectric EEG represents the most abnormal EEG pattern and indicates a recording where no convincing electrocortical activity can be recorded.

Maturation of the EEG

The normal and mainly discontinuous EEG of the extremely preterm infant is characterized by bursts of high-voltage activity alternating with periods of lower-voltage activity (the IBI). Short periods of more continuous activity can be seen also in very immature infants. With increasing maturation the EEG becomes increasingly continuous, i.e., the periods with low-voltage activity become shorter, and the duration of the bursts increases while their amplitude decreases. For quantitative purposes the duration and number of activity and the duration of IBIs are of interest. Since the IBI in healthy preterm infants contains low-voltage activity, various cuto s have been used. A commonly used definition of IBI is low-voltage activity <30 μV for >1 second between bursts of higher voltage activity (also often defined as activity above a certain level with a duration of >1 second).

Mean IBI duration decreases from 18 to 26 seconds at 21–24 gestational weeks, to 10–12 seconds at 25–30 gestational weeks, and down to 6–8 seconds at 34–36 weeks (Connell *et al.*, 1987a; Hahn *et al.*, 1989; Selton *et al.*, 2000; Hayakawa *et al.*, 2001; Vecchierini *et al.*, 2003). Prolonged IBIs are associated with brain damage and/or deep sedation, a predominant IBI >30 seconds in full-term infants is associated with poor outcome (Menache *et al.*, 2002).

The EEG waveforms, i.e., the frequency distribution, also change during maturation, although there is usually a mix of frequencies at all conceptional ages. High-voltage delta activity, and to some extent theta activity, is predominant in most preterm infants while theta and even alpha activity can be identified in full-term infants. Studies evaluating power spectral analysis and SEF in healthy preterm and term infants have shown correlations between PCA and absolute power within the theta and delta bands, respectively (Bell *et al.*, 1991). However, it was also noted that the SEF changed depending on the EEG derivation, and that preterm infants had greater intraindividual and interindividual variability in SEF values than full-term infants.

The topographical organization of the developing EEG occurs in an occipital–frontal direction. At 24–26 weeks' PCA, the activity is usually synchronous between the hemispheres, but between 26 and 30 weeks there is increased interhemispheric desynchronization, which again is followed by increasing synchronization at PCAs above 30 weeks. These EEG features are only examples of gross developmental milestones in the EEG; for more detailed information the reader is referred to other reviews (Lamblin *et al.*, 1999; Mizrahi *et al.*, 2004).

Some EEG patterns and waveforms can be used for maturational evaluation of the EEG because they are expected to appear and disappear at specific PCAs (Mizrahi *et al.*, 2004). However, the functional and anatomical correlates of most of these activities are not known, e.g., delta brush patterns, temporal theta bursts, and frontal sharp wave transients. Delta brush patterns are complexes with fast activity (alpha-beta) superimposed on delta activity, and typical of the preterm EEG. Temporal theta bursts ("temporal saw-tooth") are brief, rhythmic, 4–6 Hz transients that appear around 26 postconceptional weeks and reach a maximum at 30–32 weeks' gestation before they disappear. Temporal saw-tooth is associated with normal outcome when present at 27–30 weeks' PMA, but abnormal if present a few weeks later (Mizrahi *et al.*, 2004). Temporal sharp waves may appear during the first weeks of life in infants born at 31–32 weeks' gestation. However, if abundant, or persisting, they are associated with brain injury. Frontal sharp wave transients appear from 34 to 35 weeks' PCA and disappear around 10 weeks later.

EEG and sleep–wake states

Three sleep states can be delineated in newborn infants: quiet sleep (QS), active sleep (AS), which is also called rapid eye-movement sleep (REM), and transitional or indeterminate sleep. The di erent sleep–wake states can usually be distinguished by observations of eye movements, respiration, muscle tone, and movements. The EEG during AS is mainly continuous, and state di erentiation by EEG between wakefulness and AS is difficult without direct observation of the infant, or without recording of eye movements, which are a prominent feature of AS/REM sleep. In the full-term infant, two types of EEG can be distinguished during QS: HVS, which is a continuous pattern with slow but high-amplitude activity, and *tracé alternant*, which is a discontinuous pattern with bursts of activity mixed with periods of attenuated, low-voltage activity. Sleep–wake states are usually clearly delineated in the healthy full-term newborn infant. In preterm infant EEG, crude sleep–wake

cycling can be identified as early as 25–26 weeks' PCA, but is usually more evident by 27–30 weeks' PCA (Selton *et al.*, 2000; Kuhle *et al.*, 2001; Vecchierini *et al.*, 2003; Scher *et al.*, 2005). In the EEG, QS periods are represented by increasing discontinuity, and AS by a more continuous background pattern, resulting in cyclic variations of the EEG background. Variability of the EEG background can also be seen early in life in extremely immature infants, presumably re ecting cyclic alterations between quiet sleep and active sleep/wakefulness (Fig. 14.1).

Amplitude-integrated EEG

The amplitude-integrated EEG (aEEG) is a trend measure of the EEG which includes recording of the EEG from a reduced number of channels (usually one or two), filtering, rectifying, and smoothing of the signal before it is displayed in a heavily time-compressed manner, which makes long-term trends in electrocortical activity easily identifiable. The aEEG output is shown in a semilogarithmic fashion, which enables variations in very low EEG voltages to be more easily recognized. The method was developed in the 1960s and was initially used in the "cerebral function monitor" (CFM), which printed the resulting signal on to heat-sensitive paper usually with a paper speed of 6 or 30 cm/h (Maynard *et al.*, 1969; Hellström-Westas *et al.*, 2003). Newer aEEG monitors modify the digitally recorded and stored EEG in a similar fashion as in the early CFM, and display both the aEEG and the original EEG. The new digital EEG monitors have introduced increased exibility, which includes possibilities of also creating other EEG trends (e.g., SEF, continuity measures), and of varying the number of EEG recording channels including recording of full EEGs.

The maturational development of aEEG tracings parallels that of the EEG, which can be expected since the aEEG is derived from the EEG. Due to the semilogarithmic display of the aEEG, this method is very sensitive for showing changes in activity within extremely low voltages. Consequently, a discontinuous aEEG with interburst amplitudes below 5 μV may correspond to a suppression burst pattern when a standard EEG is recorded in full-term infants (Toet *et al.*, 2002). In the aEEG, the normal *tracé discontinu* EEG of preterm infants, with low-voltage activity during the interburst intervals, can usually be distinguished from burst suppression with mainly isoelectric interburst intervals. The *tracé discontinu* has a more variable lower margin while the burst suppression usually has a straight lower margin. When evaluating the aEEG in infants with very depressed background patterns, it is also necessary to scrutinize the original EEG to exclude interference in the trace from electrocardiogram, electronic equipment, or high-frequency ventilation.

Several studies have published normative pattern descriptions and amplitude values for normal aEEG tracings at di erent gestational ages from the extremely preterm to full-term infants (Viniker *et al.*, 1984; Thornberg & Thiringer, 1990; Burdjalov *et al.*, 2003; Olischar *et al.*, 2004). The maturational feature in the aEEG which seems to correspond best to the increasing EEG continuity with increasing maturation is a continuous increase of the minimum amplitude of the aEEG during quiet sleep.

The main features that can be extracted from the aEEG include:

- the type of background activity in terms of discontinuous/continuous activity;
- an estimation of interburst intervals or burst rate;
- cyclic variation in the background activity corresponding to sleep–wake cycling;
- the presence of EEG seizure patterns.

It should be noted that with the filtered and time-compressed aEEG, it is not at all possible to evaluate fine details of the EEG, instead the strength of aEEG is the possibility of following long-term trends in overall electrocortical activity. Also when using EEG monitoring with reduced numbers of electrodes, complete information about, e.g., hemispheric side asymmetries and synchrony, frequency content of EEG bursts, and occurrence of specific EEG features of clinical and prognostic significance, such as delta brushes, positive rolandic sharp waves (PRSWs), temporal sharp waves, or other interictal sharp transients, cannot be obtained. A full EEG should therefore be recorded in most high-risk infants who are supervised with continuous EEG monitoring.

EEG and perinatal brain injury

An acute severe insult that a ects cerebral blood ow, oxygenation, or metabolism evokes characteristic changes in the EEG. The initial EEG reaction to such insults includes amplitude depression, increased discontinuity, seizures, and loss of sleep–wake cycling. In asphyxiated infants, quantitation of EEG background can also reveal decreased EEG spectral power, mainly

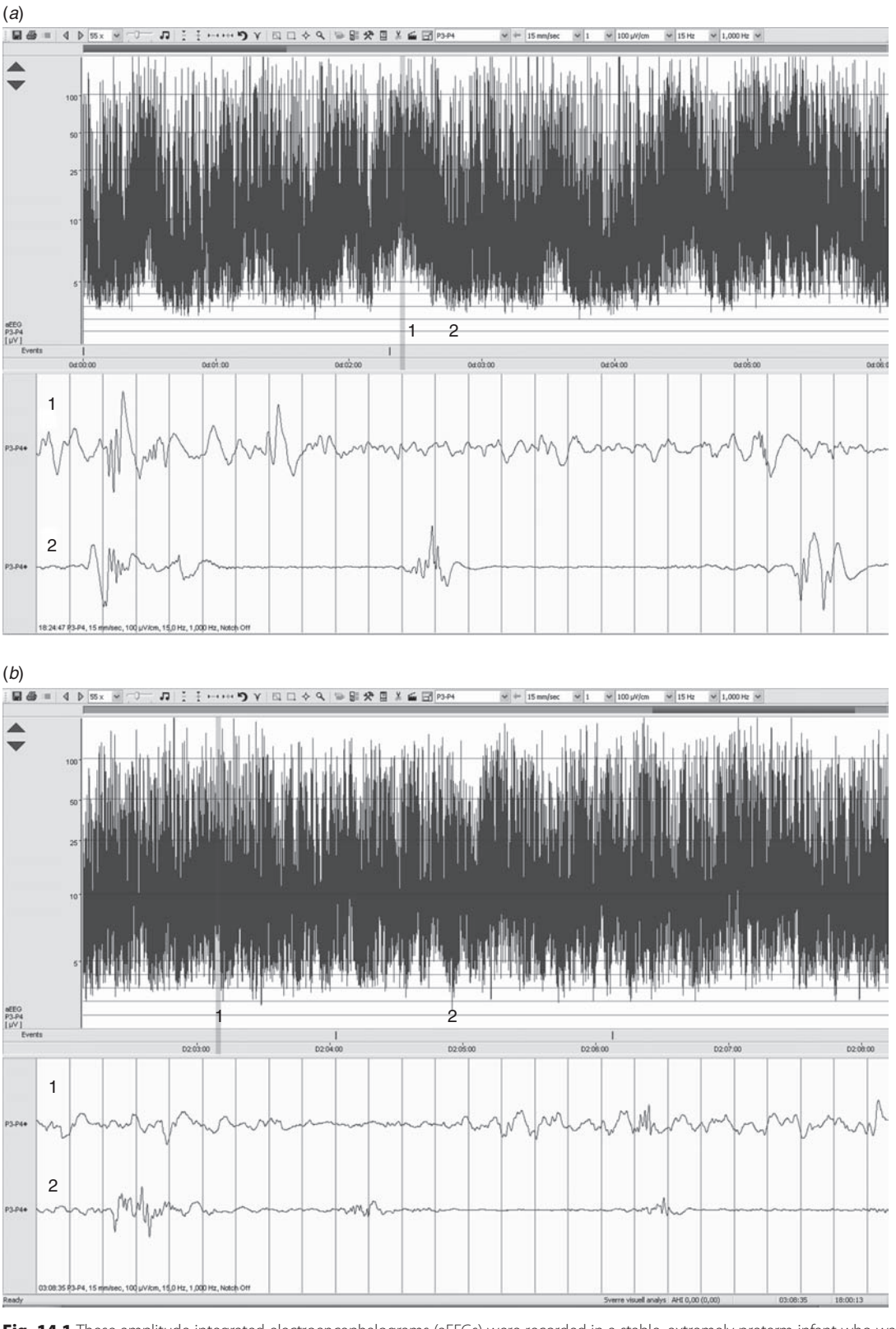


Fig. 14.1 These amplitude-integrated electroencephalograms (aEEGs) were recorded in a stable, extremely preterm infant who was born at 23 weeks and four days of gestation. Below the six-hour aEEG tracings are 25 seconds of EEG showing periods with continuous (1) and discontinuous (2) activity, respectively. (*a*) This was recorded on the first day of life, and shows a stable aEEG pattern with some short-term variability, which probably can be regarded as "normal" for an extremely preterm infant at this gestation. (*b*) aEEG of the same infant on the second day of life. Smooth cyclical variations in the lower aEEG, with a duration of 30–60 minutes, can be seen, and probably represent immature sleep–wake cycling.

within the delta frequency band (Bell *et al.*, 1990). The severity and duration of such EEG changes are directly related to the severity of the insult and also correlated to risk for persisting neurological injury. The EEG changes are usually not specific for a certain type of injury. The EEG activity recovers over time and may become normalized within a week or two even after severe insults leading to permanent injury. Watanabe and colleagues (1999) have characterized EEG "acute stage" and "chronic stage" abnormalities in newborn infants in relation to outcome. Relatively subtle "chronic stage abnormalities" may persist in the EEG when neurological damage has occurred. Such changes include disorganized background activity, maturational delay (more than two weeks), abnormal sleep–wake cycling and presence of abnormal wave forms, e.g., temporal sharp waves and PRSWs. PRSWs are markers of white matter damage and emerge one to four weeks after the injury. Presence of more than two PRSW per minute is predictive of cerebral palsy (Marret *et al.*, 1997). A quantitative measure of EEG power – the 90% SEF, i.e., the frequency below which 90% of the EEG power exists – has been shown to be closely related to white matter damage in preterm infants (Inder *et al.*, 2003). Figure 14.2 shows EEGs recorded at 40 weeks' PCA in a pair of preterm twins born at 27 gestational weeks, one with white matter damage and the other without.

"Acute stage abnormalities" can be seen in asphyxiated full-term infants, and in infants with other conditions compromising cerebral oxygenation or blood flow, e.g., cardiac malformations, or in infants developing pneumothorax, hypoglycemia and severe infections. Acute stage abnormalities can also be present in preterm infants, e.g., during development of intraventricular hemorrhages or white matter damage. These abnormalities can often easily be identified by aEEG, while evaluation of possible "chronic stage abnormalities" requires recording of a full EEG.

Seizures

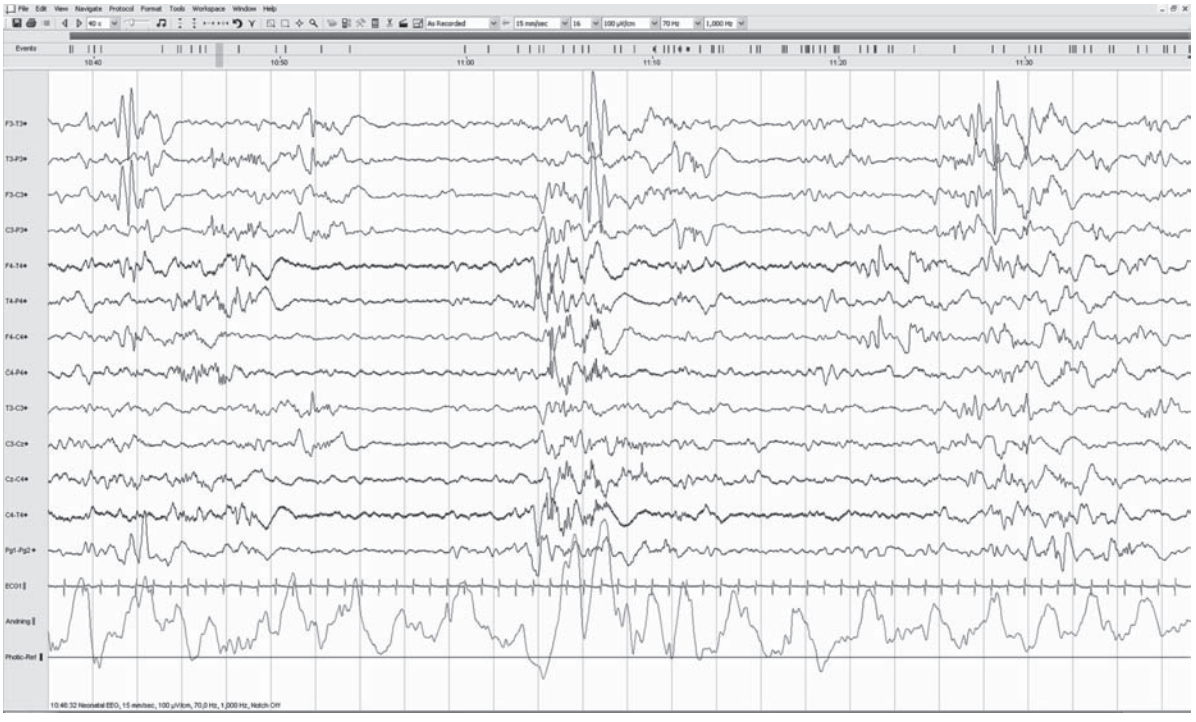
Around 2 per 1000 live born infants are diagnosed with seizures, with the risk being five- to tenfold increased in preterm infants. In some high-risk neonatal populations, e.g., in infants after cardiac surgery, up to 10% of infants may have seizures, mainly subclinical (Helmers *et al.*, 1997). Earlier studies using aEEG also showed that subclinical seizures were common (occurred in 65%–75%) in preterm infants developing intraventricular hemorrhages (Greisen *et al.*, 1987; Hellström-Westas *et al.*, 1991). Hypoxic–ischemic or hemorrhagic brain injury, hypoglycemia and metabolic diseases, severe infections such as sepsis and meningitis, congenital malformations, and maternal drug misuse are among the most common etiologies of neonatal seizures, although there are also some rare epileptic syndromes that may cause seizures in newborns (Tharp, 2002). Rare causes of neonatal seizures include pyridoxine-dependent seizures, benign familial neonatal-infantile seizures due to ion-channel mutations (potassium and sodium), and "fifth-day seizures," which are idiopathic seizures that were more common one to two decades ago (Tharp, 2002).

A seizure pattern in the EEG is characterized by repetitive waveforms that increase and decrease in frequency and amplitude with a definite onset, peak, and end. The duration varies from five to ten seconds to several minutes; ten seconds has been used as a criterion for minimum duration of a seizure in many studies. More recent data indicate that also briefer periods of repetitive activity are associated with neurological damage (Oliveira *et al.*, 2000). *Status epilepticus* is usually defined as continuously ongoing or repeated seizures with a duration of at least 30 minutes, or more than 50% of the EEG recording time.

A majority of neonatal seizures probably have durations of less than one minute, although the mean duration may depend on whether antiepileptic drugs have been administered or not. It was consequently shown in a study in which the majority of infants had received antiepileptic treatment that the mean seizure duration was less than one minute, while the average seizure duration was five minutes in full-term infants and two minutes in preterm infants in another study where around 50% of the infants received antiepileptic treatment (Clancy & Legido, 1987; Scher *et al.*, 1993). Figure 14.3 shows seizures as displayed by the aEEG with corresponding EEG in a full-term infant with seizures due to intracranial hemorrhage.

Subclinical seizure is the most common seizure-type, although many infants have a mixture of electroclinical, i.e., electrographic changes with clinical symptoms, and electrographical only, i.e., subclinical seizures (Clancy *et al.*, 1988). It is not uncommon that electroclinical seizures continue as electrographical seizures after administration of antiepileptic

(*a*)



(*b*)

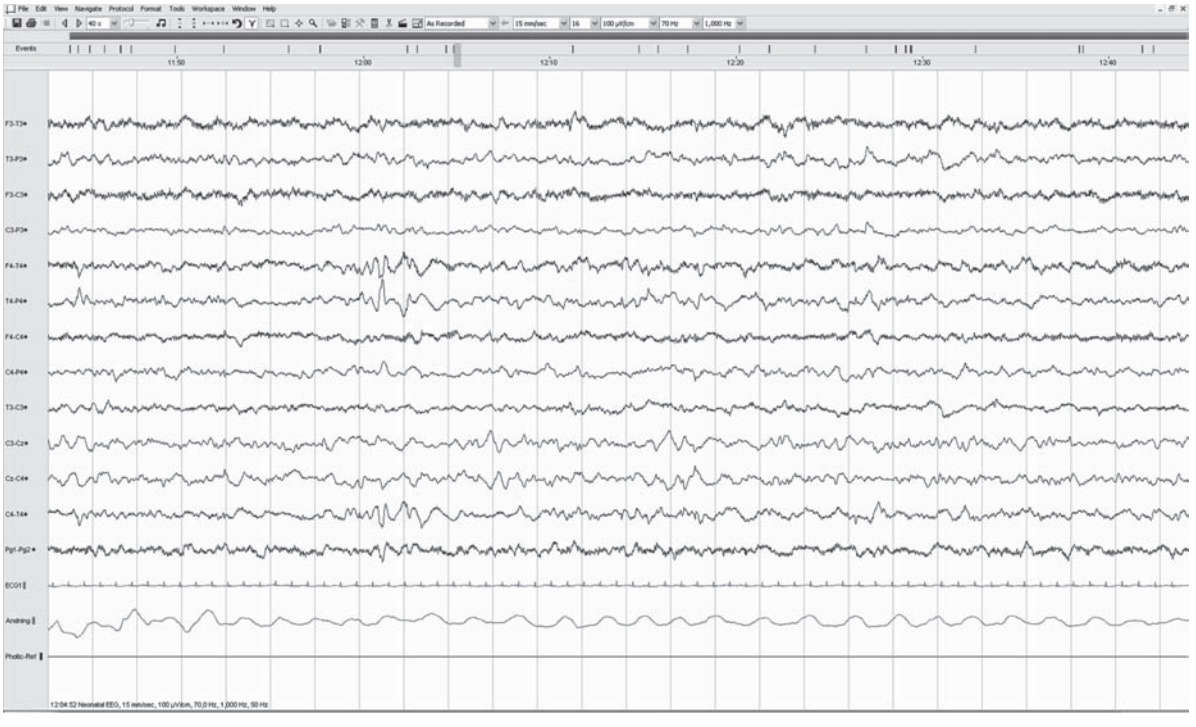


Fig. 14.2 These electroencephalograms (EEGs) were recorded at 40 weeks' postconceptional age in a pair of twins born at 27 gestational weeks. Both infants developed respiratory distress syndrome, for which they required mechanical ventilation. The clinical course was complicated in the second twin, who developed a pneumothorax, followed by a large intraventricular hemorrhage with hydrocephalus, and later need for a ventriculoperitoneal shunt. The neurodevelopmental outcome at 12 months of age was normal in the first twin, but the second twin had suspected cerebral palsy. The EEGs were recorded during quiet sleep. (*a*) In the first twin, a symmetrical continuous predominantly delta activity is seen, with occasional temporal sharp waves (normal for age). (*b*) In the second twin, the pattern is discontinuous with reduced synchrony, and with frequent independent sharp waves on both sides.

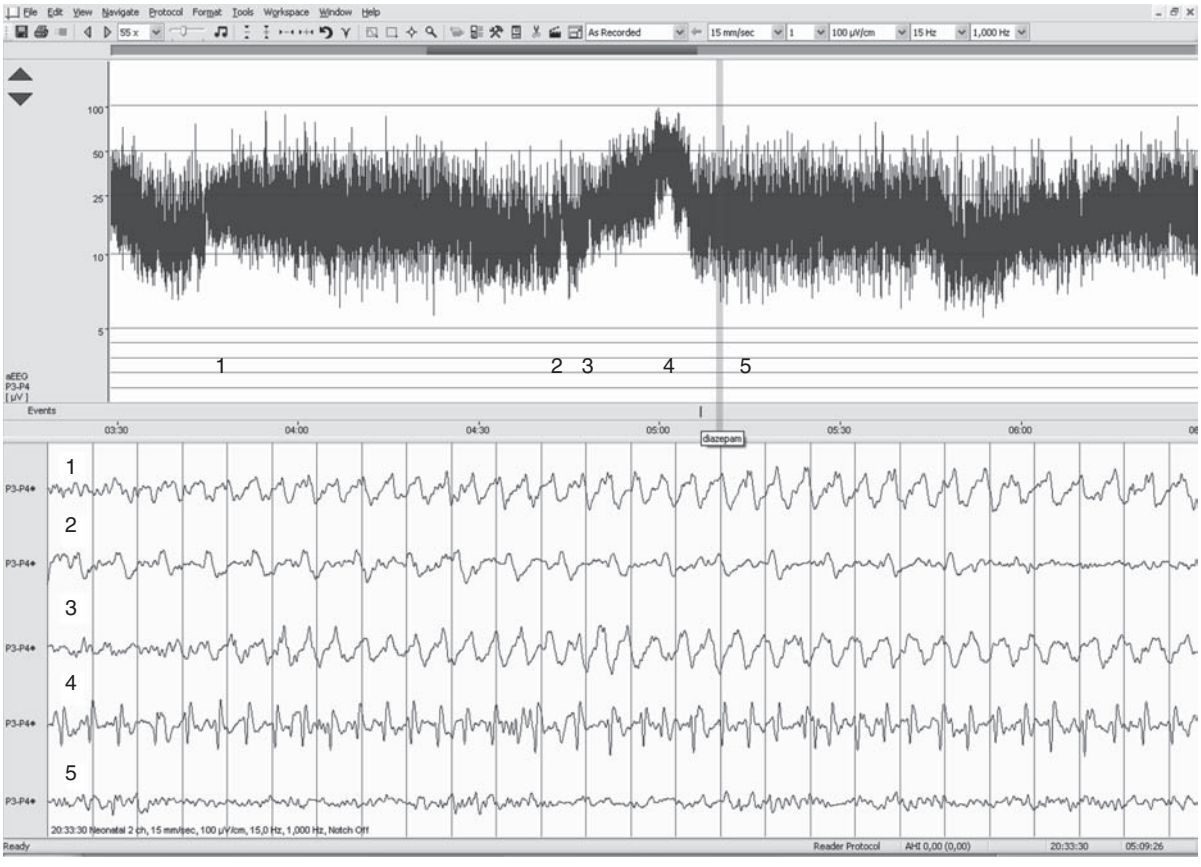


Fig. 14.3 A full-term infant developed seizures after birth and was diagnosed with a large intraparenchymal hemorrhage. The three-hour amplitude-integrated electroencephalogram (aEEG) with 25 seconds of corresponding EEG traces (1–5) below. Three very brief seizures can be seen in the aEEG (numbers 1–3) as very brief and sharp changes in the minimum amplitude. Another seizure with a duration of around ten minutes can be seen as an overall rise of the trace at "4." The overall EEG background is mainly continuous, which can be seen both in the three-hour aEEG recording with minimum amplitudes around 10 µV and the corresponding EEG at "5" (after administration of diazepam). There is a slow cyclicity in the overall amplitude indicating imminent, but not normal, sleep–wake cycling.

treatment, a phenomenon that has been called "uncoupling" (Boylan *et al.*, 2002; Toet *et al.*, 2002; Hellström-Westas *et al.*, 2003). Consequently, clinical identification of seizures by clinical observation alone is difficult and unreliable in newborn infants (Murray *et al.*, 2008). Diagnosis of epileptic seizures and evaluation of antiepileptic treatment in infants with diagnosed seizures are therefore two important indications for continuous EEG monitoring in newborn infants.

EEG and aEEG monitoring of seizures

Video EEG monitoring can probably be considered as the gold standard for diagnosing epileptic seizures, but the method may be cumbersome to apply and the interpretation needs a specially trained neurologist or clinical neurophysiologist. Video EEG monitoring has mainly been used for pediatric and adult epilepsy investigations, although some centers also use it for examination of newborn infants, especially in complicated cases. The method has not been used as an overall screening method, or brain monitor, in high-risk infants in neonatal intensive care.

Furthermore, repeated standard EEGs need to be performed since the aEEG does not give information about individual EEG waveforms or their topographical distribution during the seizures. It can be estimated that 80%–90% of seizures can be recognized with a single- or two-channel aEEG with simultaneous display of EEG (Toet *et al.*, 2002).

Most neonatal seizures start in the temporal and central areas. Preterm infants usually have a more regional seizure onset, while it is often more focal in

full-term infants (Patrizi *et al.*, 2003). The number and spatial distribution of EEG electrodes that are necessary for optimal identification and treatment of neonatal seizures is not known, although a few studies have tried to address this issue. In a study of 31 infants with seizures, only 166 of 187 seizures were identified and in one infant the seizures were not identified when the number of EEG electrodes was reduced from 19 to 9 (Tekgul *et al.*, 2005). A reduction of electrodes from 12 to 4, in another study including 32 neonates with seizures, resulted in underestimation of the number of seizures in 19 infants, and in 2 infants seizures were not identified (Bye & Flanagan, 1995). A single-channel EEG (C3–C4) also detected 78% of seizures

Seizures can be identified in the aEEG by pattern recognition, they are distinguished by a transient rise in the aEEG amplitude, usually both the upper and lower border of the trace (Fig. 14.4). Status epilepticus and recurrent seizures result in repetitive peaks in the aEEG trace, which can be described as a "saw-tooth pattern." It is not uncommon that aEEG or continuous EEG monitoring, which may run for hours and days, detects the presence of seizure activity, which is not diagnosed when intermittent standard EEG is recorded. However, suspected seizure activity in the aEEG needs to be confirmed by corresponding changes in the original EEG, since also care procedures, for example, may result in seizure-suspected changes in the aEEG pattern. Also, brief seizures or continuously ongoing spiking (e.g., periodic lateralized epileptiform discharges [PLED]) may be impossible to diagnose in the aEEG since they do not create visible transient changes in the aEEG, and for this reason the original EEG should always be evaluated (Fig 14.4).

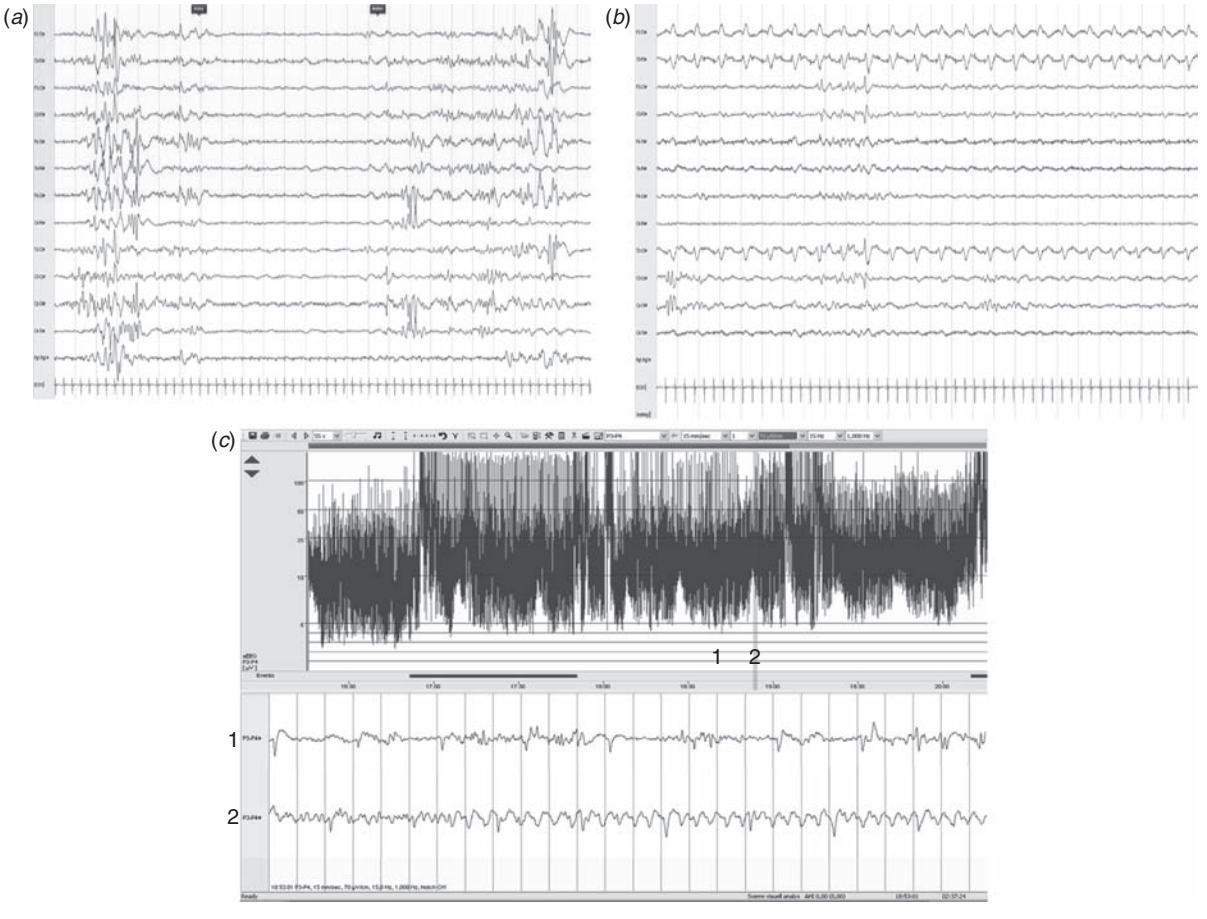


Fig. 14.4 Two parts of an electroencephalogram (EEG) and amplitude-integrated electroencephalogram (aEEG) of a full-term infant with group B streptococcal meningitis who developed seizures. The EEG shows (*a*) a discontinuous background, and (*b*) a seizure over the left temporal region. (*c*) Four hours of corresponding single-channel aEEG recorded from biparietal leads, showing a discontinuous background (1) with repeated seizures (2) with similar appearance as in the EEG.

and 94% of EEGs with seizures in a group of 125 infants with 851 seizures identified by standard EEG (Shellhaas *et al*., 2007). A probable explanation for the high proportion of seizures detected in a single channel is that a seizure, when it is focal, produces voltage gradients over the skull which can be detected with bipolar leads, provided that a large enough distance between the recording electrodes is used. The same study also compared how accurate a single-channel aEEG (also derived from C3–C4) but without access to EEG was in detecting seizures. In the aEEG without EEG only 26% of seizures were detected in 40% of the infants. The low aEEG seizure detection rate, when the EEG is not available, is comparable with the data from a previous study (Hellström-Westas, 1992). In that study, 15 high-risk neonates had single-channel or five-channel aEEGs/EEGs. Seizures developed in ten infants; five of the infants had status epilepticus, which was diagnosed by the aEEG. There were also 48 single, EEG-verified, seizures recorded, but only 15 of these seizures were possible to identify with the aEEG since they were of short duration (5–30 seconds) and did not result in clearly visible changes in the time-compressed aEEG display.

Effects of medications on the EEG/aEEG

Many infants in whom there is an indication to record EEG have received sedative or antiepileptic medications before the EEG. The evaluation of an abnormal EEG can in these infants become indecisive, since such drugs can depress the electrocortical background activity, and thereby obscure the real status of the brain. When the EEG background is normal or only slightly depressed, there is usually no problem with the interpretation. Several studies have evaluated e ects of various drugs on the neonatal EEG. Almost all such studies have been performed in ill infants who had a clinical indication for receiving a certain drug, since administration of drugs only for evaluating EEG e ects would not be ethically justified in healthy neonates.

Most sedative and antiepileptic drugs depress the electrocortical background activity, and the extent of the depression is associated with the type of medication, the dose and the time the drug was given in relation to the EEG, and the maturity of the infant (Hellström-Westas *et al*., 1988; Bell *et al*., 1993; Nguyen The Tich *et al*., 2003; van Leuven *et al*., 2004). Although not evaluated specifically, it is our impression that healthier babies respond less while more severely ill babies, and neonates with cerebral compromise have more pronounced electrocortical responses. Knowledge from continuous EEG monitoring has contributed considerably to our understanding of drug e ects on electrocortical background activity. Sleep–wake cycling is often obscured by sedatives or antiepileptic drugs in both term and preterm infants, even in the absence of evident brain damage.

Opioids such as morphine and fentanyl, administered either as bolus doses or continuous infusion, are associated with increased background depression. Although the changes usually are relatively minor in full-term and moderately preterm infants, the reactions in very preterm infants may be more pronounced and moderately discontinuous background patterns may change into burst suppression (Bell *et al*., 1993). Loading doses of phenobarbital (10–20 mg/kg) and phenobarbital concentrations within therapeutic levels seem only to moderately a ect the EEG background in full-term infants, but they increase the interburst intervals in preterm infants (Bell *et al*., 1993; Hellström-Westas *et al*., 2003). However, phenobarbital does not appear to a ect the sensitivity of the burst rates in the aEEG for prediction of outcome in preterm infants with a large intraventricular hemorrhage (Hellström-Westas *et al*., 2001). In asphyxiated full-term infants with a mainly continuous aEEG background, a phenobarbital loading dose may result in a change to a slightly, or moderately discontinuous background, whereas a comparable loading dose in severely asphyxiated infants with a mainly continuous EEG background may result in more pronounced depression, e.g., a change to burst suppression or low voltage.

It was recently shown that recovery of aEEG background after a bolus dose of sedative or antiepileptic medication takes a median of 2.5 hours, although the range may be very wide (15 minutes to 15 hours) (Shany *et al*., 2008). Moderate doses of midazolam infusion for sedation during mechanical ventilation in full-term infants is also not associated with major changes in aEEG patterns. Administration of lidocaine is often associated with EEG depression, often resulting in burst suppression. This may be an additive e ect, since lidocaine often is given as add-on antiepileptic treatment after previous administration of phenobarbital and benzodiazepines. Endotracheal administration of surfactant can result in a short, but very profound, depression of electrocortical activity which may become transiently inactive for about ten

minutes (Hellström-Westas *et al.*, 2003). The reason for this reaction is not known.

Prediction of outcome

The EEG is a highly sensitive predictor of outcome when recorded early after an insult, i.e., within the first hours and days after a severe event. As shown in several studies in full-term infants, the rate of recovery of the electrocortical background after a severe insult is associated with outcome, and the longer the EEG remains abnormal the higher the risk for subsequent adverse outcome. Pezzani *et al.* (1986) recorded EEGs during the first 24 hours of life in 80 full-term infants requiring intensive care for various reasons. Severe background abnormalities such as burst suppression with IBI greater than 40 seconds, inactive (isoelectric) tracings, and seizures were associated with poor outcome, while minor abnormalities and preserved sleep–wake cycling were associated with normal outcome or minor sequelae.

Burst suppression is a marker of severe brain damage (or deep sedation) in the full-term infant and is associated with high mortality and subsequent handicap. A nonreactive burst suppression and a predominant IBI duration of more than 30 seconds identifies infants with the poorest outcomes, and is associated with a 100% probability of death or severe neurological handicap and an 86% risk for epilepsy in survivors (Menache *et al.*, 2002). The burst suppression pattern represents a disconnection in brain circuits between the cerebral cortex and deep layers, e.g., the thalamus (Steriade *et al.*, 1994). Burst-suppression EEG is also typical of some metabolic diseases associated with high mortality or adverse neurodevelopmental outcome. Postmortem neuropathological investigations have shown direct relationships between the number of damaged neurons and the degree of EEG background abnormalities in both full-term and preterm infants (Aso *et al.*, 1989, 1993). Inactivity in the EEG correlated to widespread encephalomalacia a ecting the cerebral cortex, corpus striatum, thalamus, midbrain, and pons in postmortem studies of newborn infants (Aso *et al.*, 1989). Burst suppression was also related to multifocal severe brain damage but no damage to any specific brain structures were identified in these infants.

Intractable seizures, and seizures associated with severely abnormal EEG background patterns are usually associated with the worst outcomes (Scher *et al.*, 1993; van Rooij *et al.*, 2007). However, several factors in uence the prognosis in infants with seizures, including the etiology of the seizures and the maturity of the infant. Subclinical seizures and brief rhythmic discharges have also been reported to be associated with adverse outcome (Oliveira *et al.*, 2000).

In preterm infants both acute stage and chronic stage EEG abnormalities have been associated with adverse neurodevelopmental outcome (see below). The ability of the early EEG to predict neurodevelopmental outcome in very preterm infants is lower than in the full-term infants.

Very preterm infants may have a medically complicated course over several weeks, including development of pulmonary complications and late-onset sepsis. It was consequently shown, when serial EEGs were recorded in preterm infants, that repeated normal EEGs were associated with normal outcome. However, presence of a markedly abnormal EEG at any stage during the first weeks of life was associated with poor outcome, and this EEG was not always the first to be recorded (Tharp *et al.*, 1989).

Although EEG and aEEG are sensitive indicators of brain function, and their ability to predict outcome is high in, for example, asphyxiated infants, a combined approach including clinical neurological evaluation, imaging, e.g., magnetic resonance imaging (MRI), and other functional measures, e.g., near-infrared spectroscopy, will probably increase the precision when evaluating infants.

EEG and hypoxic–ischemic insults

An acute hypoxic–ischemic event, severe enough to compromise brain function, is associated with depression of the electrocortical activity with subsequent recovery after the insult (Watanabe *et al.*, 1999). The degree and duration of the EEG depression correlates with the severity of the insult, and the rate of EEG recovery is associated with the extent of brain damage and is predictive of neurological outcome. The EEG reaction does not only occur as a response to perinatal asphyxia, but is also a general response to hypoxic–ischemic insults which also may a ect infants with other conditions, e.g., diaphragmatic hernia, congenital heart disease, persistent pulmonary hypertension of the newborn (PPHN), and sepsis.

Perinatal asphyxia

Several studies, including one metaanalysis, have shown that outcome can be accurately predicted

from an aEEG in asphyxiated full-term infants during the first hours after birth (Hellström-Westas *et al.*, 1995; al Naqeeb *et al.*, 1999; ter Horst *et al.*, 2004; van Rooij *et al.*, 2005; Shany *et al.*, 2006; Spitzmiller *et al.*, 2007). In eight studies, included in the meta-analysis, there was an overall sensitivity of 91% (95% confidence interval [CI] 87% to 95%) and a negative likelihood ratio of 0.09 (95% CI 0.06 to 0.15) for aEEG to predict poor outcome accurately (Spitzmiller *et al.*, 2007). The authors of the metaanalysis recommended that aEEG should be part of the initial evaluation of infants with suspected hypoxic–ischemic encephalopathy (HIE). Since the aEEG recovers over time, it is most sensitive for prediction of outcome when recorded within the first 48–72 hours, although a persistently abnormal aEEG would still predict a high risk for adverse outcome after that time period (ter Horst *et al.*, 2004). The sensitivity of the early aEEG to predict outcome in moderately preterm asphyxiated infants is probably also good, but there are very few data evaluating this.

Crude prediction of outcome (healthy/not healthy) can be performed at three and six hours postnatally with an accuracy around 80% and 90%, respectively (Toet *et al.*, 1999). Recovery can also occur during the first 24 hours, and if an initial burst-suppression pattern changes to a continuous background there is a 50% chance of good outcome (van Rooij *et al.*, 2005). The postnatal age at which sleep–wake cycling appears in asphyxiated infants is also predictive of outcome, a feature that is especially useful when evaluating infants with moderate HIE. In 171 asphyxiated infants, the median time for onset of sleep–wake cycling was 7, 33, and 62 hours, in infants with HIE grades I, II, and III, respectively. In infants with HIE II, the median time for onset of sleep–wake cycling also di ered between infants with good outcome (29 hours) and infants with poor outcome (48 hours). This observation is of clinical importance since outcome in infants with HIE II is uncertain and no discriminative measures have been identified that can predict outcome with better accuracy (Osredkar *et al.*, 2005).

Both pattern recognition and amplitude-based classifications are equally sensitive for evaluation of aEEGs from asphyxiated infants at six hours postnatally, although it seems that pattern recognition may be more sensitive at three hours (Shany *et al.*, 2006). Moderate hypothermia is increasingly used for neuroprotection after perinatal asphyxia, but does not seem to in uence aEEG amplitudes, as shown in a pilot study of infants treated with extracorporeal membrane oxygenation (ECMO) and hypothermia (Horan *et al.*, 2007). However, preliminary data indicate that also during treatment with moderate hypothermia delayed recovery of aEEG background can be associated with good outcome.

Presence of seizures in asphyxiated infants, whether electroclinical and/or subclinical, does not seem to be a predictor of outcome, probably since seizures are more common in infants with moderate HIE than in infants with severe HIE. However, the duration of status epilepticus is clearly associated with outcome in infants with HIE (van Rooij *et al.*, 2007).

Perinatal stroke

The typical clinical presentation of a term infant with perinatal stroke is focal clinical seizures, appearing during the first one to two days, in an infant who is otherwise doing well. Neonatal stroke can also develop in asphyxiated infants, in dehydrated infants, and in infants with cardiac malformations. The EEG is often asymmetrical, and amplitude depression and seizures can be detected on the a ected side as well as asymmetries in sleep–wake cycling. The EEG background is also associated with outcome in these infants, an abnormal unilateral or bilateral EEG background being associated with an increased risk that the infant will develop neurological deficits (Mercuri *et al.*, 1999). Continuous aEEG monitoring is often very useful in infants with stroke, not least for evaluation of antiepileptic treatment.

Hypoxic–ischemic insults due to causes other than perinatal asphyxia

Several groups of infants who are in need of intensive care treatment have a high risk of experiencing clinical situations in which cerebral function may be compromised. Among high-risk infants are babies with congenital heart defects, congenital diaphragmatic hernias, PPHN, and infants developing pneumothorax and severe infections with cardiovascular instability. With EEG monitoring it is possible to evaluate brain function continuously in such infants, although there are few published studies of such high-risk infants.

It has been shown that the EEG recorded early is associated with neurological outcome in infants with severe respiratory failure and need for ECMO. Serial EEGs were recorded in 119 infants during ECMO; infants with two or more EEGs showing burst suppression or seizures had an odds ratio of 6.6 (95% CI 2.2 to 20.2) for adverse outcome, i.e., death, or neurodevelopmental sequelae as compared with infants without EEG abnormalities (Graziani *et al.*, 1994). Also the aEEG seems to be predictive of outcome in these infants, as shown in a study of 20 neonates treated with ECMO (Pappas *et al.*, 2006). There were no acute changes or lateralizing e ects in the aEEG during cannulation when ECMO was initiated. An abnormal aEEG predicted adverse short-term outcome, death, or moderate to severe intracranial neuropathology with 100% sensitivity, 75% specificity, 86% PPV and 100% NPV. In a study describing aEEG during ECMO combined with moderate hypothermia, there were no significant e ects from the hypothermia on the aEEG background. However, 10 of the 26 infants had moderately to severely abnormal aEEGs including three infants with seizures (Horan *et al.*, 2007). Infants with congenital cardiac malformations constitute another high-risk group for hypoxic–ischemic brain damage, and up to 10% of these infants also develop postoperative seizures (Helmers *et al.*, 1997).

In preterm infants, cardiac output and mean arterial blood pressure correlate with amplitude and continuity measures in the EEG/aEEG background (Victor *et al.*, 2006; West *et al.*, 2006b). A decrease in relative EEG delta activity and prolonged interburst intervals was demonstrated in a group of very low birthweight infants when mean arterial blood pressure fell below 23 mmHg (Victor *et al.*, 2006). It is possible that blood carbon dioxide levels, which a ect cerebral blood ow, also may in uence EEG in preterm infants (Victor *et al.*, 2005b).

White matter damage

Di use periventricular white matter damage is common in preterm infants. Cystic periventricular leukomalacia (cPVL) represents the most severe form of white matter damage and is usually associated with development of neurological handicap. There are few studies that have investigated acute changes in EEG related to white matter damage, one reason for this being the difficulty in establishing early diagnosis of white matter damage and in correlating this to clinical events and to EEG findings. Connell and colleagues (1987b) used two-channel EEG monitoring and detected EEG depression and seizures during the first days of life in preterm infants developing periventricular echodensities. Increasing severity of white matter damage in preterm infants has also been shown to correlate with decreasing SEF (Inder *et al.*, 2003). There was no di erence in SEF values between infants who were recorded during the first week of life and later, indicating that early changes in electrocortical activity are present in infants developing white matter damage.

The presence of chronic EEG changes in preterm infants in relation to outcome has been extensively studied. PRSWs, i.e., sharp transients of positive polarity over the central (rolandic) regions and appearing in preterm infants during the first weeks of life, are indicators of white matter damage. Their presence is predictive of cerebral palsy if more than two PRSWs per minute appear in the EEG (Marret *et al.*, 1997).

Okumura and colleagues (2002) investigated different types of chronic EEG changes in relation to outcome at two years of age in 183 preterm infants with gestational age less than 33 weeks. In 103 infants there were no chronic stage abnormalities present; 95 of these children had normal cognitive development and only 4 developed cerebral palsy. In 52 infants with disorganized EEG, only 50% had normal cognitive development, 39 (75%) developed cerebral palsy, and 31 (60%) had PVL on ultrasound, which was confirmed by later MRI. A dysmature EEG was found in 28 children, only 8 had normal cognitive development and 5 developed cerebral palsy. Of these children 16 had normal ultrasound, 11 had intraventricular hemorrhages without parenchymal involvement, and only 1 had PVL. Few studies have compared quantitative EEG with early imaging. In a group of preterm infants without white matter damage, who were given care according to NIDCAP or standard care, brain development was assessed by EEG coherence and MRI with di usion tensor imaging (DTI) (Als *et al.*, 2004). In the NIDCAP group, behavioral measures were better, EEG coherence between frontal and occipital regions was higher and DTI findings indicated more mature fiber structures.

EEG and hemorrhagic lesions

Birth trauma and vascular anomalies

Birth trauma severe enough to result in intracranial hemorrhages and neurological symptoms may also result in focal or generalized EEG background changes and seizures. Vascular anomalies may also result in hemorrhages with corresponding clinical and EEG abnormalities. The underlying condition in these infants usually determines the prognosis.

Germinal matrix and intraventricular hemorrhages

Several studies have described EEG changes associated with germinal matrix/intraventricular hemorrhages and hemorrhagic parenchymal infarctions in very preterm infants. The normal *tracé discontinu* background pattern is sometimes replaced by burst suppression, and seizures may be present. In infants developing hemorrhages the electrocortical background activity can be persistently depressed over the first days (Greisen *et al.*, 1987; Hellström-Westas *et al.*, 1991). The degree and duration of early EEG depression correlate with the severity of the intraventricular hemorrhage, especially the extent of the brain damage, which was further demonstrated in postmortem examinations of preterm infants with intraventricular hemorrhage (Aso *et al.*, 1993). In some studies, EEG background depression was also observed in some infants before the hemorrhage was demonstrated with cranial ultrasound (Connell *et al.*, 1987b; Greisen *et al.*, 1987).

aEEG studies from the late 1980s showed that seizures, often entirely subclinical, were present in up to 60%–70% of very preterm infants developing germinal matrix hemorrhages (Greisen *et al.*, 1987; Hellström-Westas *et al.*, 1991). To our knowledge, no later studies evaluating long-term EEG or aEEG changes in relation to development of intraventricular hemorrhage in preterm infants have been published. It is our impression that seizures now are less prevalent in preterm infants developing germinal matrix hemorrhages, but this impression could also be due to the lower incidence of hemorrhages during the past decade. No studies have specifically evaluated EEG or aEEG in preterm infants with cerebellar hemorrhages.

In very preterm infants, prediction of outcome from EEG is not possible in a way comparable with that in full-term infants, i.e., based on EEG/aEEG background patterns based on continuity, since the normal EEG background is mainly discontinuous. Instead, quantification of the degree of continuity/discontinuity can be made by estimating the burst rates or the interburst intervals. Only a few studies have evaluated the early EEG to estimate if early prediction is possible. It was shown by aEEG in infants surviving with large hemorrhages (grade 3–4 intraventricular hemorrhage) that the maximum burst count per hour during both the first and second days of life di ered between infants who died and those who survived with severe handicap and those who survived with no or moderate handicap (Hellström-Westas *et al.*, 2001). Furthermore, poor outcome (death or survival with severe handicap) was correctly predicted by a maximum burst count of less than 130 per hour in 68% and 78% of infants at 0–24 hours and 24–48 hours, respectively.

The cuto at 130 bursts/h should be regarded as arbitrary, since it could be dependent on the type of monitor that was used, and most of the infants in the study above also received phenobarbital. In the same infants the lowest burst count over one hour was also evaluated, but did not di er significantly in relation to outcome probably because this measure is also sensitive to administration of morphine and sedative medications. Quantification of continuity has also been performed in aEEG by estimating the amount of activity that is present below a certain amplitude level. For the old analog CFM, 3 μV has been used, and in newer digital monitors several cuto values, e.g., 50 μV and 100 μV, have been evaluated (West *et al.*, 2006a). In a group of extremely preterm infants, with median gestational age of 25 weeks, it was consequently shown that the percentage of continuity during the first week of life correlated with both cranial ultrasound findings and outcome at 2 years of age (Hellström-Westas *et al.*, 1991). Presence of seizures was not associated with outcome in these infants. However, presence of sleep–wake cycling at the end of the first week of life was associated with better outcome. Presence of sleep–wake cycling in the aEEG has also been associated with good outcome in extremely preterm infants with smaller or no intraventricular hemorrhages (Hellström-Westas *et al.*, 1991; Kuhle *et al.*, 2001).

There are no specific chronic EEG changes associated with intraventricular hemorrhage in preterm infants. It was previously believed that PRSWs were

related to intraventricular hemorrhage. However, it was later demonstrated that PRSWs are markers of white matter damage, which also may develop in infants with intraventricular hemorrhage.

EEG and infectious diseases

Neonatal meningitis and meningoencephalitis due to bacterial or viral infections are often associated with irritability, seizures, lethargy, and other neurological symptoms.

Multifocal periodic patterns in the EEG have been described in neonatal herpes simplex meningoencephalitis, otherwise there are no specific EEG patterns associated with neonatal infections, and the most common findings are nonspecific abnormalities in electrocortical background activity and presence of seizures. In a study estimating the predictive value of EEG in 37 newborn infants with bacterial meningitis, a moderately to markedly abnormal EEG was found to be predictive (sensitivity 88%, specificity 90%) of death or survival with poor outcome (Klinger *et al.*, 2001).

EEG and metabolic diseases

Hypoglycemia is the most common metabolic cause of brain dysfunction in newborn infants. However, there are only a few studies evaluating electrocortical function during hypoglycemia in newborn infants. In a study of moderately hypoglycemic infants of diabetic mothers, there were no changes in aEEG background pattern (Stenninger *et al.*, 2001). This is not surprising since early hypoglycemic changes in adults include increases in lower EEG frequencies, and if comparable relatively subtle changes are present in newborns, they will not be possible to detect with aEEG, although could possibly be detected with EEG power spectral analysis. A few case studies in newborn infants have demonstrated aEEG background depression and seizures with severe hypoglycemia (Hellström-Westas *et al.*, 2003).

High bilirubin levels may increase the amount of slow wave activity in the EEG, and bilirubin encephalopathy associated with development of kernicterus can be associated with seizures (Gürses *et al.*, 2002). Infants with metabolic diseases often develop electrocortical background changes and seizures, although most of the EEG abnormalities are unspecific and not related to a certain disease. However, burst suppression seems to be a consistent EEG feature for some severe encephalopathies, e.g., nonketotic hyperglycinemia, hemimegalencephaly, and Ohtahara syndrome, and urea cycle defects are often associated with very intense seizure states.

Conclusion

The EEG is a powerful tool for evaluation of brain function in newborn infants and is also useful for prediction of later neurological outcome. Soon after an insult, the degree of overall EEG background depression and the time to recovery indicate the extent of brain damage. Presence of seizures and postnatal age when sleep–wake cycling appear are factors that also may in uence on outcome. At later stages, more subtle chronic changes appear in preterm infants with brain damage. The EEG varies with the maturation of the infant, but the chain of EEG events after various insults is similar. Both acute and chronic EEG changes are mainly nonspecific as regards the di erential diagnoses. Continuous EEG monitoring, and especially the aEEG, has proven to be a valuable complement to intermittent standard EEG recordings in the neonatal intensive care unit.

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Chapter 15

Emergence of spontaneous and evoked electroencephalographic activity in the human brain

Sampsa Vanhatalo and Kai Kaila

Introduction

The adult human brain is estimated to contain about 10^{11} neurons with about 1000–10 000 synapses targeting each of them. This means that within each person's skull, there is a genuine microcosmos, in which the number of key elements (the ~10^{14} synapses) far exceeds the number of stars in our galaxy. The mere numbers above, however, provide only a faint picture of the true complexity of the brain, which is more accurately reflected in the microanatomical and functional specificity of the neuronal connections. The structural and functional specificity of wiring requires precise targeting of presynaptic endings to their proper postsynaptic locations at the subcellular level, and this has to be achieved for both local and distant connections in the course of brain development. In addition, the brain retains a high degree of structural and functional plasticity throughout an individual's lifetime, and therefore the wiring of neuronal networks is subject to control systems that maintain and also modify existing connections (Pascual-Leone *et al.*, 2005). The plasticity of the brain is at its highest during development, and there is a massive literature describing various kinds of "sensitive" and "critical" periods during which the initial, preestablished connections show a heightened sensitivity to activity-induced modification (Katz & Crowley, 2002; Hooks & Chen, 2007).

Our genes total about 20 000 in number, and this has often been contrasted with the brain's complex phenotype to provide an argument against "genetic determinism" in brain development. However, this is not a valid comparison at all. The genes in brain cells are best thought of as data-storage systems for controlled synthesis of proteins, where selective reading of information (selective gene expression) during various stages of development is absolutely crucial for neuronal maturation and differentiation. Notably, gene expression is under the influence of neuronal activity, and changes in protein synthesis caused by selective activation of gene expression are known to form the basis of a large spectrum of plasticity phenomena, with timespans ranging from a few hours to days and years. Hence the classical nature–nurture debate can be considered a historical remnant based on ignorance, rather than a discussion that would address a real neurobiological problem.

An overwhelming amount of data, dating back to the classical work of David Hubel and Torsten Wiesel (see Katz & Crowley, 2002; Hooks & Chen, 2007), has shown that activity does, indeed, play a critical role in brain development. Much of this work has been carried out using sophisticated electrophysiological techniques to make invasive recordings in vivo as well as in experiments on isolated brain tissue and on acutely isolated or cultured neurons. The electrophysiological observations are often effectively combined with immunohistochemical and biochemical experiments. For obvious reasons, experiments of this kind are not possible on the human brain, with the exception of work on brain tissue that has been surgically removed for therapeutic reasons.

Faced with the above problems in gaining "first-hand" information on the basic modes of operation of human neurons and neuronal networks, the only feasible strategy is to make meaningful extrapolations from animal experiments. However, the term *meaningful* can easily lead us into a logical minefield, because it requires an objective strategy that would

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allow us to evaluate the scope and limitations of extrapolations from animal experiments to the human condition. The present chapter will focus on the analysis of the developing human electroencephalogram, and we will make an attempt to present some strategies and (at this time) a limited conceptual vocabulary for translational studies between animals and human beings.

The information content of the neonatal electroencephalogram depends on the progress of basic neurobiology

Textbooks of basic neurobiology usually have a very brief section on electroencephalography (EEG) (sometimes none at all), which is somewhat surprising when considering the long history of EEG, starting from the pioneering studies on animals by Richard Caton and on humans by Hans Berger. The main reason for the lack of enthusiasm about EEG studies by the neurobiological community is probably that, until recently, the data provided by EEG have been largely phenomenological, with a very superficial link to the overwhelming amount of information on the basic mechanisms whereby neurons communicate. Despite the tremendous number of neurons that make up any functional circuit in the brain, the information content, from the experimenters' point of view, of single-cell and local field-potential (invasive "micro-EEG") recordings in animals has been much higher than the (at first sight rather enigmatic) traces that are produced by scalp-recorded EEG.

However, times are changing, and the various kinds of neuronal network mechanism underlying EEG signals are being identified – with the bulk of research done on animal models in vivo and ex vivo. This kind of work spans all the hierarchical levels of organization of the brain, from molecules to neurons and to networks. Hence, the EEG is becoming less and less phenomenological, and mechanistic conclusions can now be drawn on the basis of these noninvasive recordings. This will strongly increase the utility of EEG in both basic and clinical neuroscience.

Regarding the neurophysiological basis of the early patterns seen in the immature human EEG, a number of observations made in animal experiments have influenced current thinking. First, there is little doubt that neuronal activity has a major influence on the wiring of the nervous system. Second, it is clear that the most fundamental signaling mechanisms in mature and immature neurons can be qualitatively different. A point of focus here are the signaling modes of the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the adult brain, which has depolarizing (sometimes even excitatory) actions in practically all central neurons at an early stage of development (Blaesse *et al.*, 2008). This has a number of implications for the causal analysis of neuronal network events as well as for the evaluation of drug actions. Third, it has become clear that all structures in the immature central nervous system produce endogenous or spontaneous, "self-organized" network activity (Sipilä & Kaila, 2008). This is also true for sensory systems, where spontaneous and spatially correlated activity is generated before these systems are even capable of receiving stimulation-induced excitation. For instance, in the visual system, spontaneous activity in the retina ("retinal waves") is generated before the functional maturation of photoreceptors, and these waves are crucial for the appropriate wiring (including retinotopy at the level of the cortex) of the visual system (Katz & Crowley, 2002; Hooks & Chen, 2007).

The early spontaneous activity most likely plays a number of roles in brain development. In view of the discontinuous and highly synchronous nature of these patterns, they are, in principle, eminently suited for triggering the release of trophic factors, including brain-derived neurotrophic factor (BDNF). Notably, BDNF is released in an activity-dependent manner from target neurons to provide trophic support for incoming afferents, and, not surprisingly, cells that fail to establish synaptic connections within a specific time window of their development will die (Lessmann *et al.*, 2003; Innocenti & Price, 2005). Controlled cell death (apoptosis) is a major factor that contributes to the formation and maintenance of neuronal networks.

Spontaneous activity is also likely to be needed to provide a temporal code for side-by-sidedness of the neurons involved, which is a basic requirement in the establishment of topologically faithful projections in the brain. In the visual and somatosensory system, for example, perceiving the external world (vision) or one's own body scheme (somatosensation) are dependent on the accurate maintenance of spatial relationships within the neuronal pools that code for adjacent stimulus locations.

Endogenous versus evoked activity

The distinction between "spontaneous/endogenous" and "exogenous/evoked" activity is not as clear as one might perhaps expect. Starting from the work of William Preyer in the 1880s on chick embryos in ovo, it has become evident that embryonic motor movements are generated in all vertebrate species, and their spatiotemporal patterns undergo an elaborate differentiation (de Vries & Fong, 2006; Lüchinger *et al.*, 2008). These myoclonic twitches are known as "baby kicks" in utero, and they are the predominant pattern of motor activity in preterm babies. This kind of self-generated, spontaneous movements will provide a rich source of somatosensory information that is likely to affect the wiring of the participating networks (Schouenberg, 2008). In this case, the source of movement and the associated proprioceptive activity are endogenous, while the tactile stimuli (resulting from the baby's touching itself or the amniotic membrane bordering the wall of the womb) are best classified as exogenous. Interestingly, the myoclonic twitches have their origin in bursts of activity that are generated in the spinal cord, and coordinated by the brainstem (Blumberg & Lucas, 1994).

A further problem in categorizing central events into endogenous or evoked is that, for instance, much of the slow large-scale activity in the early developing cortex seems to be generated in an endogenous manner, but activity patterns with similar characters can be evoked by sensory stimulation (Khazipov *et al.*, 2004; Milh *et al.*, 2007). This topic has further ramifications, because even in the adult brain, sensory-evoked responses are largely shaped by the spontaneous activity in intrinsic circuitry in the thalamus and cortex. From an experimental point of view, a *tour-de-force* test for a genuinely "endogenous" pattern of activity is to demonstrate that it can be observed in isolated, living neuronal tissue. Such experiments were initially done on invertebrates (which contributed to the demolishing of the Watson–Skinner type of behaviorism), but more recently a variety of sophisticated patterns of activity have been observed in brain slices from both neonatal and adult animals (Sipilä & Kaila, 2008). It is hardly necessary to emphasize the impact of this kind of work, which permits the use of the whole armamentarium of modern neurobiology in solving fundamental problems related to brain development.

From a clinical point of view, it is important to note that abnormalities in the patterns of spontaneous activity are likely to lead to defects in brain development. Apart from genetic factors that are implicated in disturbances in the formation of synapses in several developmental diseases (e.g., Down syndrome, Fragile X), it is clear that various kinds of intrauterine and extrauterine factors can lead to changes in endogenous activity patterns and thereby to pathophysiological changes in the structure and functions of evolving circuits.

Structural milestones in the ontogeny of the human cortex

In light of what has been said so far, it is evident that in any attempt to understand the properties of EEG activity, it is imperative to have knowledge about the structural properties of the neuronal circuits that generate the scalp-recorded signals. While the basic molecular, cellular and network mechanisms related to human brain development are largely based on information from animal experiments (see above), detailed structural studies can be often performed in a satisfactory manner on postmortem human tissue. In addition, modern noninvasive neuroimaging techniques have made it possible to study in vivo a wide range of questions that influence current thinking about the physiological and pathophysiological mechanisms of early brain development (Hüppi & Dubois, 2006; Prayer *et al.*, 2006; Perkins *et al.*, 2008; see also Rados *et al.*, 2006). A number of major advances have been recently made in studies on the development of neuronal connectivity in the immature human brain (for a review, see de Graaf-Peters & Hadders-Algra, 2006; Kostović & Jovanov-Milosevic, 2006; Kostović & Judas, 2007). It appears that many salient aspects in the development of brain activity can be linked to the overall histological development of human brain structures, and especially to the emergence of neuronal connectivity within and between them.

Considerable data are currently available on the development of human sensory organs and their peripheral nerve pathways, as well as on the development of thalamocortical connections. However, there is surprisingly little knowledge about how the central connections develop from the sensory organs to their corresponding thalamic nuclei. A recent study with neuronal tracing techniques on postmortem human

fetal brains showed that the retinogeniculate pathways of the visual system (Hevner, 2000) develop during the second trimester of pregnancy. Indirect functional evidence from the somatosensory system suggests also that the spinothalamic pathways are present before the growth of the thalamocortical connections (Vanhatalo & van Nieuwenhuizen, 2000; Fitzgerald, 2005). Functional development of brainstem structures during gestation is much less understood. However, the presence of distinct, brainstem-generated movement patterns (de Vries & Fong, 2006; Lüchinger *et al.*, 2008) as well as the emergence of vigilance state cycles at around the onset of the third trimester (Kozuma *et al.*, 1998) suggest that widespread brainstem-linked neuronal connections are functional long before they become effectively connected with cortical networks. Hence, the developmental changes of both spontaneous and evoked cortical activities are mostly dependent on the maturation of the thalamocortical and corticocortical connections. Formation of these networks progresses in three major, somewhat overlapping phases (see also Fig. 15.1).

First, the newly migrated neurons form local neuron clusters, future cortical columns, where some hundreds of neurons communicate among each other within a millimeter scale. This phase takes place mainly during the second trimester.

Second, long-range connections are formed between thalamus and cortex, as well as between distant cortical sites, allowing concerted interactions across and between hemispheres (for detailed reviews, see Kostović & Jovanov-Milosevic, 2006; Kostović & Judas, 2007). This long-range network formation proceeds in phases so that the growing axons first reach the subplate layers in their cortical target areas, followed by a gradual translocation of axonal terminals to the deepest cortical layers, and further into their final targets in cortical layer IV. This vertical translocation of cortical afferent terminations is seen as distinct changes in the development of evoked activity (see below and Vanhatalo & Lauronen, 2006). The first long-range connections to be established are thalamocortical projections. They arrive at the subplate zone by the end of the second trimester, and their progression into sensory cortices is mostly completed at around the thirty-second week. The long-range corticocortical connections within (Judas *et al.*, 2005; Hüppi & Dubois, 2006) and between hemispheres via callossal projections (Jovanov-Milosević *et al.*, 2006) appear a few weeks later, beginning their contact formation with the targets at around weeks 29–30. The major interhemispheric connections are established a few weeks later, while the intrahemispheric connectivity evolves during a much longer time window that spans the last trimester of pregnancy and up to the first years of life.

Third, histological studies on human fetal development have demonstrated that the adult intracortical compartmentalization of distinct afferent versus efferent neuronal layers (i.e., short-range connections) is not yet developed in the preterm brain (reviewed in Ramakers, 2005; Kostović & Judas, 2007). For instance, the major afferent cortical layer IV is prominent in the early preterm motor cortex, but it disappears during the neonatal period (Kostović & Judas, 2007). The emergence of short-range corticocortical circuitries is hence intimately linked to the structural differentiation of cortical layers and their columnar specifications.

A prominent feature of brain development is the presence of transient structures and circuitries, that are known to disappear soon after term age. Perhaps the most important of these structures is the subplate, a very dense and thick neuronal network under the developing cortex proper (Kostović & Rakic, 1990; Kostović & Judas, 2006). The thickness of the subplate in humans may be up to four times that of the cortical plate (Kostović & Rakic, 1990), and therefore it is a salient structure also in magnetic resonance (MR) images (Rados *et al.*, 2006; Perkins *et al.*, 2008). The subplate is known to serve as a waiting zone and a guidance hub for the incoming cortical afferents from the thalamus and from other cortical areas (Kostović & Judas, 2007). It acts as an early relay zone for sensory afferents coming to the cortex, as well as an orchestrating machinery for the early endogenous cortical activity and its structural substrates (Dupont *et al.*, 2006; Kanold & Shatz, 2006; Vanhatalo & Lauronen, 2006; Kanold, 2009). Furthermore, during early development, long-range projections in the corticospinal tract are transiently bilateral (Eyre, 2007), and the interhemispheric corticocortical projections show a marked transient exuberance with a significantly denser and larger innervation in target areas compared with the mature circuitry (Innocenti & Price, 2005).

It is notable in the human context that: brain development proceeds over a significantly extended

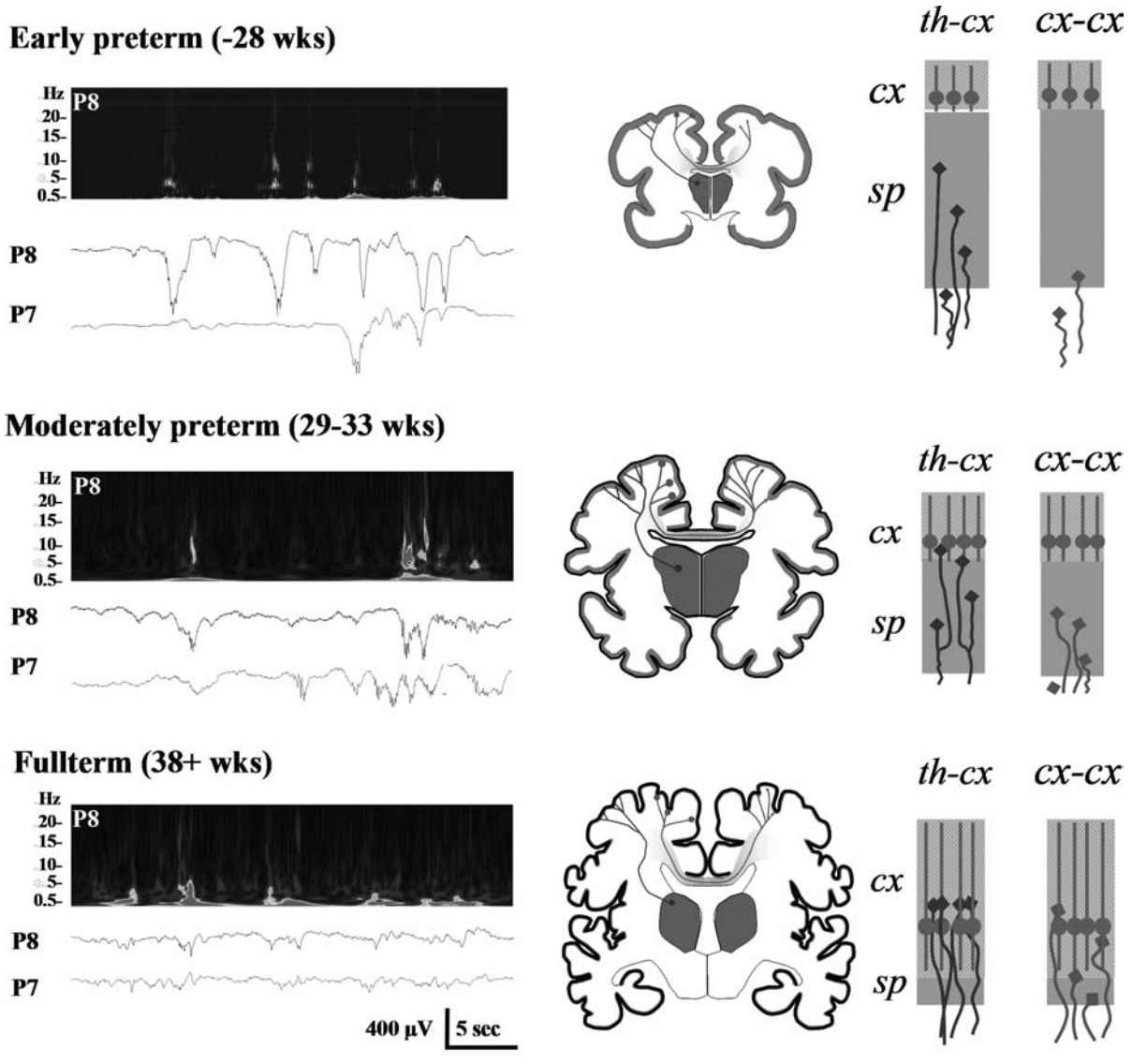

Fig. 15.1 (See color plate section.) Major developmental milestones of cortical function (left side) and structure (right side) of the human preterm baby. Left side shows one EEG trace (unpublished data) from each parietal area lobe (P8 is right and P7 is left; Fz reference). In addition, a time-frequency representation of the P8 trace is shown to demonstrate the multiband nature of the EEG events (see main text). In the early preterm baby, the EEG activity consists mainly of brief, monophasic, high-amplitude events, which are relatively independent in each cortical area. The thalamocortical (th-cx) connections are just arriving at the subplate–cortex layers, whereas the corticocortical (cx-cx) connections have not yet grown. In the moderately preterm baby, the EEG activity consists of a little longer but still rather high-amplitude, mono- or biphasic events, which gradually become more synchronized between distant cortical sites. The thalamocortical connections are now being established in the cortex proper, and the corticocortical connections are growing into the subplate and deep cortical layers of the target areas. In the full-term baby, the EEG activity consists of relatively long and lower amplitude events, which are relatively synchronized within and between the hemispheres. In addition, there is abundant ongoing (continuous) activity during the intervals between the discrete events. The thalamocortical connections are now mostly established in the cortical layer IV, and the corticocortical connections are innervating their final target zones and layers in the target areas.

period of time when compared with other mammals, including nonhuman primates (Kostović & Judas, 2006, 2007); there is a long coexistence of overlapping structural entities (e.g., thalamo-subplate and thalamocortical loops) during and after the third trimester; and the establishment of distinct developmental stages may differ by several months between adjacent brain areas. An interesting implication of the extended period of brain development and of the area-specific developmental heterogeneity is that

brain activity during the last trimester and the early neonatal period represents a continuously changing mixture of multiple mechanisms. For instance, formation of the sensory thalamocortical afferents and the corticocortical circuitry within the sensory cortices commences at the onset of the third trimester, whereas the corresponding development in the frontal cortical areas starts several months later and continues long into infancy (Ramakers, 2005). Needless to say, this kind of heterogeneity is of fundamental significance when considering the interpretation of early EEG activity from both a basic science and clinical point of view.

Functional milestones in the ontogeny of the human EEG

From the above short description of structural brain development, it is obvious that the EEG recorded in preterm and full-term babies reflect the activity of both transient (e.g., subplate) and permanent (progressively developing) circuitries. Moreover, due to the absence or immaturity of the main thalamocortical and corticocortical circuitries implicated in the generation of EEG activity seen at later stages, it is imperative to identify mechanisms that are unique to the preterm brain. A recent model for integrating structure and function in the neonatal human brain (Vanhatalo & Kaila, 2006) will be described below. We believe that an approach of this kind is likely to provide fruitful platforms for further, physiologically oriented and/or translational studies. We will start with a brief review of the basic characteristics and of the ontogeny of the human EEG, followed by a description of our model for its interpretation.

Neonatal EEG recordings have been routinely performed from preterm and full-term babies since the early 1950s (reviewed in Lamblin *et al.*, 1999). The available literature is almost exclusively based on studies where, for practical reasons, recordings are limited to a few electrodes that are placed over each hemisphere and used as bipolar derivations. The EEG signals have been traditionally amplified using a limited frequency response that cuts off low-frequency activity below about 0.5–1.0 Hz. Furthermore, the EEG has been combined with polygraphic channels in order to gain an improved identification of vigilance states (Lamblin *et al.*, 1999; Scher, 2004). Based on this approach, there is an extensive and primarily descriptive literature on human EEG activity from the earliest ages of viability (reviewed in Lamblin *et al.*, 1999). There are several important conclusions that can be based on these studies. However, the limited number of channels and in particular the limited bandwidth of the recordings (Thordstein *et al.*, 2005; Vanhatalo & Kaila, 2006; Vanhatalo *et al.*, 2008), have led to a situation where much of the information content of the early EEG activity is completely lost.

It is currently accepted that the early development of brain activity proceeds at a very similar rate irrespective of whether the baby lives in or out of the womb (Lamblin *et al.*, 1999; Abrahám *et al.*, 2007). This conclusion is based on a large amount of data demonstrating that the maturational stage of EEG is essentially similar at a given postconceptional week. For instance, a healthy baby born at 32 weeks has an EEG during the eighth postnatal week that is very similar to the EEG of a baby born at 40 weeks. This makes sense from an evolutionary point of view, because it means that the early human brain development is by and large set free from the unpredictable variation in environmental influences (see also the section "The neonatal intensive care unit as a developmental environment," p. 239). It is also worth noting that evaluating the "maturity of the EEG" with regard to the baby's conceptional age is a key point of analysis in clinical EEG routines. While some specific details in the EEG development have been reported to show differences that are associated with an early birth (see e.g., Conde *et al.*, 2005), the large number of uncontrollable confounding factors (comorbidities and selection of EEG features of interest) in studies of this kind raise questions about the validity of the conclusions.

The preterm EEG consists of discrete events

Early forms of EEG are mostly composed of events (see Table 15.1) rather than genuine oscillations (Buzsaki & Draguhn, 2004; Steriade, 2006). The prevailing approach to EEG, dominated by work on older subjects, is so heavily biased by "frequency thinking" (i.e., categorizing various kinds of activity in terms of the standard EEG frequency bands), that the preterm and neonatal EEG is often erroneously viewed as composed of independent oscillations. Various frequency components can, of course, be observed in any biological signals, including neonatal

EEG. However, the basic building blocks, typically complex events, of preterm EEG are important to recognize and acknowledge as such to enable its structured analysis and comparison with existing experimental literature. Using EEG with DC-coupling (Vanhatalo *et al.*, 2005a), we showed recently that cortical activity in preterms is dominated by large spontaneous activity transients (SATs; originally termed "slow activity transients", see Vanhatalo *et al.*, 2005b). In EEG recordings carried out using conventional recording bandwidth (AC-coupled EEG) with high-pass filtering, these large events are strongly distorted and, based on their higher-frequency components, they have been termed "spindle bursts" in animals and "delta brushes" in humans (Dreyfus-Brisac, 1964; Khazipov *et al.*, 2004; Khazipov & Luhmann, 2006; Milh *et al.*, 2007).

The traditional view of neonatal EEG activity has its main emphasis on the visually apparent developmental transformation from "discontinuous" to "continuous" EEG (Dreyfus-Brisac, 1964; Lamblin *et al.*, 1999; Scher, 2004). This means that the most preterm human EEG is considered highly "discontinuous" with only short epochs of activity interposed between long periods of silence, and the silent periods are reduced and finally vanished at around the full-term age. On top of this general scheme, numerous papers have identified various types of "graphoelements" (i.e., EEG events that are defined by their visual appearance; see Table 15.1) which undergo more specific developmental changes (Lamblin *et al.*, 1999). It is notable, however, that the multiplicity of graphoelement-based concepts has likely arisen from uncontrolled variability in the recording and analysis techniques (see also Table 15.1). Hence, the neonatal EEG has remained a surrogate measure with a clearly suboptimal sensitivity in detecting or predicting various, milder neurocognitive defects.

As mentioned above, we have recently proposed some novel technical and conceptual approaches in order to increase the information content, and thereby the clinical and scientific utility of the neonatal EEG (Vanhatalo & Kaila, 2006). Based on technical improvements related to the recording methods and data analysis (Michel *et al.*, 2004; Vanhatalo *et al.*, 2005a, 2008), it has become possible to detect brain activities that are directly comparable with observations from parallel, invasive experiments carried out on experimental animals in vivo (Meyerson, 1968; Khazipov *et al.*, 2004) or in vitro (Sipilä *et al.*, 2005; Dupont *et al.*, 2006; Kanold & Shatz, 2006; Sipilä & Kaila, 2008). This has offered a possibility to construct a simplified analysis framework that is a rough model of the preterm and full-term EEG. The model is based on two principal observations (for a more detailed description of the model, see Vanhatalo & Kaila, 2006):

1. The most salient features in the EEG throughout the preterm period are large-scale network events – the SATs (Vanhatalo *et al.*, 2005b). These long-lasting, large-amplitude events are undoubtedly the undistorted equivalents of the various delta-related graphoelements in the neonatal EEG literature (see also Table 15.1). Due to the ambiguities in the earlier EEG waveform categories, we recently proposed the more generic term SAT to refer to EEG events that in the frequency domain are characterized by the presence of multiple frequencies within the range of 0.1–30 Hz. Hence, they are also called "multiband events" from a more technical point of view (Vanhatalo *et al.*, 2005b and Fig. 15.1). More recent work in humans and in various animal models (Meyerson, 1968; Khazipov *et al.*, 2004; Khazipov & Buzsaki, 2009) has suggested that SATs most likely represent a family of network events characteristic of immature brain circuitries (see above). The available data suggest that SATs are mostly limited to neuronal ensembles that are still in the process of establishing their connectivity; they are based on self-organizing (de la Prida *et al.*, 2006; Sipilä & Kaila, 2008), spatiotemporally distinct bursts (Vanhatalo & Kaila, 2006; Vanhatalo *et al.*, 2008) and they can be generated by the cortex in isolation or in response to inputs from the underlying subplate (see below; and Dupont *et al.*, 2006) or deeper brain structures (Vanhatalo & Kaila, 2006).

   Based on the existing human literature, it appears that SATs are already observed at a conceptional age of about 23–24 weeks (Lamblin *et al.*, 1999; Vanhatalo & Kaila, 2006), i.e., at the time when the earliest premature babies survive in neonatal intensive care units (NICUs). They are first concentrated over the sensory and associative cortices in the post-central areas, but they become more widespread later in development (Dreyfus-Brisac, 1964; Lamblin *et al.*, 1999). A significant

Table 15.1 EEG terms, group features and their definition

EEG terms	Group feature	Definition and comments
Delta waves from 0.3 Hz to 2 Hz Delta-brush Delta crest Spindle-shaped bursts of fast activity Rapid rhythm Rapid bursts Spindle-like fast Fast activity at 14–24 Hz Ripples of prematurity Positive slow wave	Events with coinciding low and high frequency activity	Definition: Events with simultaneous slow and fast components. Comments: The nondistorted EEG correlate (see Figs. 15.1 and 15.2) of this cortical activity has no or barely any oscillatory component at delta frequencies. The higher frequency activity appears as a few successive cycles at about 5–30 Hz. These events are clearly not spindles akin to the sleep spindles later in life Cortical activity of this type does probably form a physiological entity (SAT) with some variations depending on, e.g., the context (endogenous vs. evoked), vigilance state, level of brain maturation, and cortical area
Temporal sharp transients Frontal sharp transients (*encoches frontales*) Anterior slow dysrhythmia	Spikes and other "fast" transients	Definition: An EEG graphoelement that is visually "sharp" or "slow" at the conventional clinical time scale, and with a limited time window of appearance during development Comments: In several phenomenological studies, attempts have been made to find out whether, or when, these EEG events should be considered as a sign of brain pathology. The limitations of the conventional EEG techniques preclude a reliable identification of their source(s) or underlying brain mechanisms The relation of these events to SATs remains to be examined
Midline frontal theta/alpha bursts Temporal saw-tooth Temporal theta bursts or theta bursts Rhythmic sharp theta activity Sharp theta rhythm on the occipital areas of prematures	Oscillations at defined frequencies	Definition: Intermittent EEG waves with an apparent frequency at "theta" or "alpha" range Comments: Despite a similar frequency range, it is most likely that the brain mechanisms generating theta (or even less alpha) are different from those known to generate oscillations at these frequencies in older children and adults (cf. mu rhythm and sleep spindles, which have similarities in frequency range, spatial location, and age range of occurrence, and yet are viewed as distinct brain activities). Moreover, the current convention of using a suboptimal electrode spacing precludes a valid spatial estimation of their sources. It is also notable that many EEG studies have ignored the concurrent slower activity, making it possible that at least some of these could represent activity in the SAT category Despite the relatively bulky literature, the above problems make it hard to conclude which of these activities should be recognized as neurophysiologically distinct
Tracé discontinu, Tracé alternant Mixed activity (*activité moyenne*) – Low voltage fast – High voltage slow	Terminology for the general EEG pattern	Definition: Overall appearance of the EEG tracing during different vigilance states and at different conceptional ages, based on a purely visual inspection of the EEG trace with a standardized time-amplitude scaling Comments: The subjective, visual analysis of continuity, flatness, oscillatory speed, or amplitude of the "general" activity is physiologically ill-defined and ambiguous These patterns are readily explained on the basis of our two-component analysis framework (see Fig. 15.3 in Vanhatalo & Kaila, 2006)

SAT, spontaneous activity transient.

proportion of SATs become roughly coincident between hemispheres very early (around 30 weeks of gestation; Fig. 15.1). A more precise and more consistent temporal synchrony of SATs arises only later, starting at around 35 weeks of conceptual age, paralleling the appearance of the interhemispheric connections (Jovanov-Milosević *et al.*, 2006; Kostović & Judas, 2007). On the basis of animal studies, it can be concluded that the interhemispheric co-occurrence of SATs is highest in homologous areas, and that this simultaneity is not fully dependent on callosal connections (Meyerson, 1968). Therefore, the apparent synchrony (i.e., coincidence) of SATs between

brain areas does not require a precisely timed triggering mechanism. The manifestation of SAT waveforms in the scalp EEG recordings is also influenced by the macroscopic developmental changes (i.e., the emergence of cortical gyri before full-term age), which likely increases variability in EEG waveforms regardless of whether and how the cortical network mechanisms change (see also Fig. 15.1). It is also possible that future translational research with invasive recordings experimental animal models will identify more specific, physiological (as opposed to graphoelement-based) subcategories in the early EEG activity.

2. During brain development, the frequency of occurrence of the discrete SAT-type events declines, and there is a gradual increase in a more continuous, oscillatory EEG activity, both in humans and in experimental animals (Meyerson, 1968; Gramsbergen, 1976; Lamblin *et al.*, 1999; Scher, 2004; Hellström-Westas *et al.*, 2006). The generation of this *ongoing* EEG activity requires that sufficiently large cortical networks become activated via thalamocortical or corticocortical connections. As already mentioned above, animal studies have shown that ongoing, higher frequency activity arises in parallel with the maturation of interneuronal GABAergic signaling (Long *et al.*, 2005; Sipilä & Kaila, 2008). Histological studies on the human brain suggest that interneuronal networks mature over a long time period in infancy (Kostović & Judas, 2007). This is consistent with the idea that the generation of ongoing, adult-type higher frequency activity (alpha, beta and gamma), believed to be essential for many cognitive functions (Buzsaki & Draguhn, 2004; Uhlhaas & Singer, 2006), is not observed in the EEG during the preterm period. It evolves gradually to replace all SAT-type activity after the neonatal period. In contrast to the temporally rough timing mechanisms in SAT activity, the ongoing cortical activity (see below) is based on oscillations in distributed neuronal networks that need to operate at a high temporal precision (Buzsaki & Draguhn, 2004; Steriade, 2006). Here, we wish to reemphasize that while SATs do contain frequencies in the delta, alpha, and beta range, these are brief transient episodes that are physiologically distinct from the canonical, "ongoing" delta, alpha, and beta oscillations seen in the mature brain.

Sensory-triggered/evoked events

A large number of in vivo and in vitro animal studies have elucidated the mechanisms of cortical activation by thalamocortical afferents during different phases of ingrowth and stabilization of thalamocortical connection. The intracortical current source density profiles have been studied with direct electrical measurement (Friauf & Shatz, 1991) and by using voltage sensitive fluorescence imaging (Higashi *et al.*, 2002, 2005). From a clinical point of view it is notable, again, that information of this kind can only be obtained from invasive recordings in animal models. However, the main cortical mechanisms of evoked responses are not only highly conserved between species, but also across sensory modalities (e.g., somatosensory, visual, auditory), making it feasible to deduce some of the main principles of operation on the basis of interspecies and intermodal comparisons. For a simplified model of how developmental changes in the thalamocortical connections are reflected in the cortical responses, see Vanhatalo & Lauronen (2006).

In the earliest stages (until about 26 weeks of conceptional age), the most advanced thalamocortical fibers make contacts with the subplate zone only. Activation of thalamocortical pathways (i.e., evoked responses) at this stage will lead to a widespread, prolonged excitation of the subplate layer (between 100 ms and 1000 ms). The subplate, in turn, may subsequently induce activity in the overlying cortical plate (Dupont *et al.*, 2006; for human studies see Hrbek *et al.*, 1973; Milh *et al.*, 2007; Vanhalato *et al.*, 2009b, as well as Fig. 15.2).

Later in development (at a conceptional age of 26–32 weeks), there is a gradual translocation of thalamocortical fiber terminals from the subplate into the deepest cortical layers, and further into their final destination in layer IV in cortex proper. This process is reflected in the gradual change from the slow, subplate-induced cortical responses to the much faster (down to tens of milliseconds), direct intracortical responses seen in full-term babies and older subjects. In invasive animal experiments, this vertical change in the evoked response depth profile is also seen as a reversal of polarity of the fastest components (Vanhatalo & Lauronen, 2006). The development of the fast cortical evoked responses continues further into infancy (Geneva *et al.*, 2002; Lauronen *et al.*, 2006), together with the elaboration

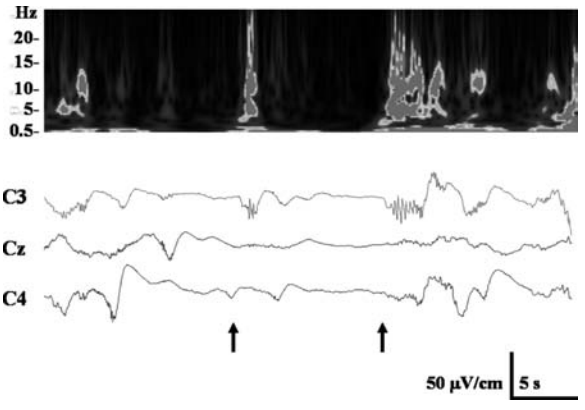

Fig. 15.2 (See color plate section.) Cortical somatosensory response in a preterm baby (conceptional age, 30 weeks). Tactile (brush) stimulation of the right palm leads to a long-lasting (one to three seconds) activity transient within the somatosensory cortex of the contralateral (left; C3) hemisphere, whereas no comparable activation is seen in the more medial or ipsilateral cortical areas (Cz and C4, respectively). In contrast, all these cortical areas exhibit SAT events, which in the time-frequency (wavelet) representation looks very much like the evoked activity. EEG traces are shown in a current source density (Laplacian) montage (unpublished data).

of short-range corticocortical circuitry (Innocenti & Price, 2005; Ramakers, 2005; Kostović & Judas, 2007).

Given the considerations above, it seems that there are two distinct, developmentally overlapping responses to sensory stimuli in the preterm and full-term brain: the "slow responses" mediated by the excitation of subplate with subsequent induction of slow, SAT-related events in the cortical networks (see below); and the conventional "evoked responses" mediated by direct inputs from the thalamus into the layer IV of cortex proper. The most salient change in the human recordings is the disappearance before term age of the slow response that is characteristic of the youngest subjects, as shown in detail by Antonín Hrbek and co-workers (e.g., Hrbek *et al.*, 1973, 1977; see also Milh *et al.*, 2007). It is notable in this context that the responses from the activation of subplate and deep cortical layers are primary responses at this age, and they arise from transient structures during a specific developmental period. Hence, they should not be confused with the much studied late responses (such as late somatosensory-evoked potential [SEP] components or P300) observed in older subjects.

For obvious technical reasons (Vanhatalo *et al.*, 2005a; Vanhatalo & Kaila, 2006; Vanhatalo & Lauronen, 2006), there is scarce literature on sensory-related brain events in preterm babies, whereas their counterparts have been extensively studied in several animal models (see references in Vanhatalo & Lauronen, 2006). These animal studies have demonstrated further that the (rapid and slow) components of cortical responses do not only arise from different cortical depths, and show different temporal profiles (see Bernhard *et al.*, 1967; Molliver, 1967; Persson, 1973; Verley, 1977, as well as Fig. 15.1 in Vanhatalo & Lauronen, 2006), but they also exhibit differential sensitivity to various physiological and pathophysiological factors. For instance, the slow component shows a remarkably higher sensitivity to interstimulus interval (Bernhard *et al.*, 1967; Molliver, 1967; Meyerson, 1968; Persson, 1973; Verley, 1977) and to asphyxia (Meyerson, 1968). Moreover, it is intriguing to note in this context that the subplate is known to be remarkably sensitive to hypoxic–ischemic brain injury (McQuillen & Ferriero, 2005). These observations together suggest that the basic characteristics as well as abnormalities of slow responses at early preterm ages may provide clinically important information about the functional status of subplate, which is believed to be crucial for a proper establishment of thalamocortical connectivity (Kanold & Shatz, 2006; Kostović & Jovanov-Milosević, 2006; Kostović & Judas, 2007; Kanold, 2009).

Interactions between spontaneous and evoked events

The similarities between the slow-evoked responses and the "spontaneous activity" described above (especially SATs, see above) raise the possibility that they belong to the same category of cortical activity. The sensory-evoked slow events are mostly confined to the primary sensory area of the given sensory modality (e.g., occipital for vision or parietal for sensory; see Discussion in Hrbek *et al.*, 1973). While many or most SAT events in the cortex may be triggered by endogenous mechanisms (i.e., spontaneously) in the subplate, some SAT-related events might be activated by sensory impulses via excitation of the subplate zone. From this perspective, there is no clear-cut distinction between "spontaneous" and "evoked" activity; SAT-like events can be triggered by an intrinsic (intracortical) mechanism, or by input from sensory systems which in itself can be spontaneous or stimulus-induced.

The "immature slow responses" to subcortical (or peripheral) activation are especially intriguing in the view of their potential role in development of long-range connectivity in the brain. Given the spatial large-scale characteristics of SAT activity, these events are endowed with key properties for developmental mechanisms whereby "neurons that fire together" would "wire together" over long distances in a hebbian manner (Katz & Crowley, 2002; Hooks & Chen, 2007).

Taken together, the available evidence suggests that the development of thalamocortical (or corticocortical) connections requires neuronal activity both for defining proper connections, and for the mere survival of these neurons (see above). This activity-dependent phase of thalamocortical development relies initially on the endogenously generated, intermittent activity (e.g., SATs), which must arise somewhere in the thalamus or in the brain areas projecting to the thalamus. Current experimental literature does, indeed, present ample evidence for the idea that thalamic and brainstem networks, as well as sensory organs exhibit spontaneous activity patterns that are implicated in the development of thalamocortical and corticocortical pathways (see above, and Momose-Sato *et al.*, 2007; Pangratz-Fuehrer *et al.*, 2007). Hence it is plausible that these subcortical brain areas and sensory organs do actively participate in pacing, i.e., in "endogenously evoking" cortical activity (Vanhatalo & Kaila, 2006). From the perspective of the early developing cortex, this kind of input may therefore arise in the underlying subplate, in any connected subcortical structure (e.g., thalamus or brainstem), or in sensory systems.

The neonatal intensive care unit as a developmental environment

A frequent theme of debate in preterm care is the NICU versus the womb as developmental environments, and on factors that are known or believed to be "optimal" for proper brain development. Concerning sensory stimuli, it is often assumed that the womb is a "stimulus-poor environment," and that babies born prematurely are exposed to an artificially "enriched environment" during their stay in NICU. There are myriad views and opinions on what are optimal qualities of somatosensory, visual, and auditory stimuli for the fetus and the preterm baby. However, most of this literature is based on beliefs (and good intentions) rather than on physiological data, and as will be pointed out, current neurobiological evidence supports the view that much of this debate is probably irrelevant.

As reviewed above, the existing evidence is consistent with the idea that, during late human pregnancy, sensory activity is rich and it is mainly generated by the fetus itself. For instance, the level of structural and functional maturation of the visual system in full-term neonates is achieved independently of any external visual input. The maturation of somatosensory systems also relies on sensory input that is generated by a somewhat more complex mechanism: the fetus exhibits an extensive repertoire of movements generated by spontaneous activity patterns in the spinal cord and brainstem (also called "general movement patterns" in clinical practice; Lüchinger *et al.*, 2008). These movements will generate rich, spatially coordinated patterns of somatosensory (tactile) inputs to the developing brain (Schouenborg, 2008). Notably, fetal tactile receptors are optimized to intra-womb inputs of this kind, since the skin receptors are mostly low-threshold mechanoreceptors (Fitzgerald, 2005). Recent animal work has shown that distortion of tactile stimulation of this kind will result in altered connectivity in the somatosensory system (Schouenborg, 2008).

It is also well appreciated from everyday experience that a third-trimester human fetus does react to sounds that are transmitted from the environment. However, it is important to note here that sound intensity in the frequency range that is relevant for the human auditory system is attenuated by up to 30–40 dB at an air–fluid interface (i.e., between the air and abdominal tissue). This basic physical constraint is often completely ignored in studies on fetal hearing, although it clearly questions an important role of the extrauterine (as opposed to intrauterine) sound environment in the fetal development of hearing.

Given the considerations above, it is obvious that cortical organization during late pregnancy depends on afferent input, and that the innate neural mechanisms in the fetus are perfectly optimized to generate this input in the uterus. This is in sharp contrast with the common belief that the intrauterine environment would be poor, and that the extrauterine environment (e.g., the NICU) would be rich in terms of sensory input to preterm baby brain. No doubt, the NICU environment generates various types of highly

unnatural stimuli relative to what the baby would experience in utero. For instance, the preterm babies are subject to frequent blood sampling with subsequent inflammatory reactions, they are exposed to relatively loud sounds related to monitoring devices, and they experience significantly distorted patterns of somatosensory feedback after spontaneous body movements. In addition, there are also several other factors (e.g., application of drugs, and changes in respiratory status) that may affect both spontaneous and evoked brain activity during the NICU period. All these exogenous factors are obviously different from what the baby would experience in utero. Indeed, some semi-empirical clinical paradigms have been developed to deliberately modify the types of sensory stimuli associated with NICU treatment (Pierrat *et al.*, 2007). Such approaches are apparently successful in reducing overall stress levels and, perhaps mainly, in raising the caretakers' attention to the well-being of the babies.

Clinical implications/conclusions

The etiology of a disease refers mainly to its causal background, and it is hard to see how any evidence-based therapy could be properly designed in the absence of causal knowledge. Integration of current knowledge on the development of human brain structure and function is opening new opportunities in the design of specific diagnostic and monitoring strategies for the detection of brain disturbances. For instance, prior studies have shown that hypoxic–ischemic or other intracranial lesions in preterm babies do often include the subplate (see McQuillen & Ferriero, 2005 and references therein), and/or the subcortical white matter (Judas *et al.*, 2005; Hüppi & Dubois, 2006; Prayer *et al.*, 2006; Perkins *et al.*, 2008). In light of data on the specific contributions of these structures to the preterm EEG, it is becoming possible to study their pathophysiology in a relatively specific manner. Neurobiological information on the basic mechanisms underlying brain activity leads to an opportunity to explore the causal mechanisms of various brain disease states and enables an objective evaluation of putative therapeutic regimens. Such vistas are especially attractive nowadays when all new clinical practices are scrutinized in the framework of evidence-based medicine.

Genuine translational work is of course based on information transfer between basic scientists and clinicians. However, even within a given project, this transfer should be both continuous and bidirectional – not simply "from the bench to the bedside." In our own research program, we attempt to identify and characterize a given clinical problem in physiological terms, and then go to the basic laboratory level to set up an animal model where specific hypotheses derived from the particular clinical problem can be tested. Currently, our translational studies include work on the generation and putative therapies of febrile seizure and, more generally, on the role of malfunctions in the regulation of brain carbon dioxide and pH in the induction of seizure activity (Schuchmann *et al.*, 2006). Another translational research theme is the excitatory actions of GABA in the immature hippocampus (Sipilä & Kaila, 2008), which is based on neuronal uptake of chloride that leads to a depolarizing GABAergic current (Blaesse *et al.*, 2008). It has been frequently suggested that the immature, excitatory mode of GABA action is important in the generation of neonatal seizures. Interestingly, in the rat hippocampus in vitro and in vivo, the excitatory actions of GABA can be blocked by bumetanide (Sipilä *et al.*, 2006), a widely used diuretic that is also known to inhibit chloride uptake in cortical neurons. However, the available data on bumetanide actions (Kilb *et al.*, 2007) do not support the idea (Dzhala *et al.*, 2005) that this drug holds promise for the treatment of neonatal seizure activity (cf. Vanhalato *et al.*, 2009a). Nevertheless, the lack of effective drugs in the treatment of neonatal seizures is one of the major problems in current pediatric neurology, and it is obvious that in this context, future advances and breakthroughs are likely to be made on the basis of a wide spectrum of parallel studies on in vitro and in vivo animal models in combination with state-of-the-art EEG recordings from human neonates.

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Section 4
Chapter
16

Clinical aspects

Infection, inflammation, and damage to fetal and perinatal brain

Gennadij Raivich and Donald M. Peebles

Introduction

Perinatal brain injury is a common cause of cerebral palsy and other neurological disabilities that affect two per 1000 live-births (Stanley & Watson, 1985), an incidence that has remained static over the past 20 years (Pharoah *et al*., 1998; Colver *et al*., 2000). Although a longstanding opinion, first proposed by William Little in 1861, suggested that events during labor were entirely responsible for cerebral palsy, there is a growing body of epidemiological evidence arguing for significant involvement of infection and infection-associated neural inflammation in mediating damage to fetal and neonatal brain. Moreover, in discussing the role of infection, it is also important to differentiate between three different pathogenetic entities: first, injury due to direct infection as in meningitis or encephalitis; second, synergistic interactions between systemic, extraneural infection and hypoxic–ischemic insult, compounding the latter; and lastly, fetal or maternal infection that occurs outside the brain, but with effects on postnatal brain development.

Congenital infections and central nervous system toxicity

During gestation, many microorganisms can infect the fetus, causing severe birth defects as well as mental retardation. On the whole, pre- and perinatal infections account for 2%–3% of all congenital anomalies (Stegmann & Carey, 2002). Neurotropic infections can occur throughout pregnancy and during birth (França & Mugayar, 2004), but the peak incidence and occurrence of neuromorphological and developmental abnormalities depends on pathogen type.

Toxoplasma gondii

Following infection with the *T. gondii* parasite, maternofetal transmission increases steadily the later the maternal infection occurs during pregnancy. Overall, it rises from 15% at 13 weeks to 44% at 26 weeks, and 71% at 36 weeks and reaching a peak just before birth (Thiébaut *et al*., 2007). At the same time the risk of intracranial lesions after maternal seroconversion drops by almost fourfold, and that of eye lesions by a factor of two with increasing gestational age. If the rates of maternofetal transmission are compounded, it suggests that immature neural and retinal tissue is particularly susceptible to damage in the event of fetal systemic infection with *T. gondii*, and that this susceptibility decreases at later gestations (Thiébaut *et al*., 2007).

Listeria monocytogenes

Like *T. gondii*, *L. monocytogenes* shows a similar preponderance of third trimester infections. However, the outcome of bacterial *L. monocytogenes* infection is much more severe. About 20% of perinatal listerial infections result in stillbirth or neonatal death (Doganay, 2003). Common complications also include premature labor and neonatal meningitis or encephalitis with subsequent neurological sequelae (Svare *et al*., 1991; DiMaio, 2000). Very early diagnosis and efficient antimicrobial treatment of listeriosis during pregnancy can result in the birth of a healthy infant, but on the whole, *L. monocytogenes* infections are acutely life-threatening, both for the fetus and the mother.

Syphilis

The majority of infants with congenital syphilis are infected in utero, but the newborn can also be infected

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by contact with an active genital lesion at the time of delivery (Chakraborty & Luck, 2007). The risk of vertical transmission of syphilis from an infected, untreated mother is known to decrease as maternal disease progresses, from 70% to 100% in primary syphilis, 40% for early latent syphilis to 10% for late latent disease. Approximately half of the cases of untreated fetal infection result in miscarriage, abortion, or stillbirth, and another quarter in premature delivery or low birthweight. Interestingly, most of the affected newborns appear asymptomatic at birth but develop symptoms similar to secondary syphilis in adults by one to two months. Prompt administration of antibiotic treatment prevents the appearance of long-term complications (Sangtawesin *et al.*, 2005).

Rubella

Over the past four decades, prenatal infections with German measles or the rubella virus have become comparatively rare in industrialized countries, due to the thorough immunization effort in infants, adolescents, and women planning to conceive who show insufficiently high antibody titer. The virus is highly teratogenic in early pregnancy. In a study on pregnancy complications following a recent rubella epidemic in Brazil, maternofetal transmission occurred in approximately half of the 33 first trimester patients, with miscarriage or intrauterine death in around three-fifths of the infected fetuses, and severe malformations, including brain damage, in the other two-fifths (Andrade *et al.*, 2006). Associated brain defects include anencephaly, hydrocephaly, and T2 periventricular and subcortical hyperintensities supportive of periventricular leukomalacia and intraventricular calcifications (Sugita *et al.*, 1991; Takano *et al.*, 2006).

Maternal rubella in the second trimester appears to lack any effect, possibly due to the much lower rates of maternofetal transmission (Tang *et al.*, 2003). Thus, in the Brazilian epidemic study that identified 27 second trimester patients, no abnormalities were found on ultrasound, prenatal, or postnatal echocardiography, newborn funduscopy, or infant brainstem evoked response audiometry (Andrade *et al.*, 2006). Anti-rubella IgM antibodies were negative in all of the newborns from mothers infected in the second trimester. Probably due to transplacental transfer, IgG antibodies were detected in some cases up to 3 months of age, at a decreasing level. However, none of these children who were transiently positive for rubella IgG had shown clinical symptoms at birth or up to 3 months of age.

Importantly, even children born without major apparent handicaps following early gestational (i.e., first trimester) rubella are at a markedly increased risk of schizophrenia spectrum disorders, a decline in IQ from childhood to adolescence, and increased premorbid neuromotor and behavioral abnormalities (Brown *et al.*, 2001). Interestingly, this applied to a cohort that showed no major physical handicaps with the exception of deafness (in many cases), and without severe intellectual impairment (IQ>70). Moreover, schizophrenia patients with congenital rubella frequently showed large areas of periventricular deep white matter T2 hyperintensities on nuclear magnetic resonance (NMR) that persisted into adulthood (Lane *et al.*, 1996). These NMR abnormalities differentiated them from control schizophrenia patients without fetal rubella infection, and suggest that defects in corticocortical and corticobasal ganglia axonal connections can provide a strong biological basis for a subpopulation of patients in this psychiatric disease (Brown, 2006; Patterson, 2007).

Congenital cytomegalovirus infection

Cytomegalovirus (CMV) infection is a leading cause of intrauterine infection and brain damage in children. Primary infection can occur in as many as 2% of previously CMV-negative, pregnant women (Yow *et al.*, 1988) and serological or culture evidence of intrauterine CMV infection has been reported in about a quarter of these cases, with no predilection to a specific stage of embryonic or fetal development (Stagno *et al.*, 1986). Among infected neonates, 90%–95% are asymptomatic at birth, but almost 30% develop late complications in the first year of life (Stagno *et al.*, 1986; Yow *et al.*, 1988). In a recent ultrasound study, combined with fetal blood IgM and amniotic fluid polymerase chain reaction (PCR) diagnosis, all fetuses with confirmed intrauterine transmission showed an abnormal pattern of periventricular echogenicity (Malinger *et al.*, 2003). In addition, more than half had echogenic intraparenchymal foci and ventriculomegaly, with some also displaying intraventricular adhesions, periventricular pseudocysts, sulcation and gyral abnormal patterns, hypoplastic corpus callosum, cerebellar and cisterna magna abnormalities, and signs of striatal artery vasculopathy. On NMR, periventricular, deep subcortical white matter T2 hyperintensities are common and

show considerable similarity to those with congenital rubella (Takano *et al.*, 2006).

Herpes varicella zoster infection

Maternal varicella infection during the first 20 weeks of pregnancy is associated with 1% neuromorphological abnormalities and 2% total congenital abnormalities (Pastuszak *et al.*, 1994). In both cases, this is an approximate twofold increase in risk of serious abnormalities compared with uninfected controls. Similar rates of incidence for congenital varicella (2%–2.5%) were also reported by Paryani and Arvin (1986) and Enders *et al.* (1994), with about a quarter of fetuses showing signs of previous exposure to systemic infection following maternal varicella infection in the first or second trimester, based on IgM and T cell response, and with approximately 10% of those showing congenital varicella syndrome (Paryani & Arvin, 1986). In the very large prospective study in Germany and UK on congenital abnormalities following maternal varicellaemia and herpes zoster, with approximately 1700 participants summarized in the Enders *et al.* report, the peak incidence of congenital varicella syndrome (CVS) occurred during the second trimester (13–20 weeks). First trimester infections were associated with a fivefold lower rate, and those in the third trimester with no central nervous system (CNS) complications. Nevertheless, the lower rates in the first trimester could have been masked by a comparatively high rate of miscarriage (Paryani & Arvin, 1986). Importantly, maternal herpes zoster was not associated with fetal varicella infection, possibly due to the absence of high levels of infectious viruses in the maternal bloodstream (Paryani & Arvin, 1986; Enders *et al.*, 1994).

Herpes simplex and human immunodeficiency virus infection

Varicella, herpes simplex type 2 and human immunodeficiency virus (HIV) are frequently, but not exclusively, transmitted at birth, causing either acutely life-threatening neonatal herpes or varicella, or, in the case of HIV, a chronically fatal disease that includes a strong neurodegenerative component (Vazeux, 1991; Griffith & Booss, 1994; Steiner *et al.*, 2007). Importantly, in all three cases, this mode of transmission can be efficiently suppressed by prenatal and perinatal application of antiviral drugs (Marculescu *et al.*, 2006).

Other viral infections affecting the fetal CNS – poliomyelitis, Japanese encephalitis, West Nile virus

In the case of poliomyelitis, reports on abortions, stillbirths, and congenital polio, primarily in late pregnancy, were published before the era of widespread immunization (Bates, 1955). On the whole, the overall rate of vertical transmission was comparatively low (Ornoy & Tenenbaum, 2006). Infection with Japanese encephalitis in the first and second trimester has been associated with miscarriage; third trimester infection did not appear to have an adverse outcome on pregnancy (Mathur *et al.*, 1985). In the case of West Nile virus (WNV) encephalitis, at the moment, there is only a single report of an infection during the twenty-seventh gestational week, the infant showing chorioretinitis and scarring, cystic destruction of cerebral tissue (as evidenced on magnetic resonance imaging [MRI] of the brain), and laboratory evidence of congenital acquired WNV infection (Alpert *et al.*, 2003).

Neonatal meningitis

High mortality and frequent neurological sequelae are characteristic of neonatal meningitis. Bacterial infection is responsible for a majority of the cases. Roughly a third is due to neurotropic viruses, particularly herpes simplex type 2; fungal meningitis is rare (Hristeva *et al.*, 1993). Group B streptococci clearly predominate in early-onset bacterial meningitis (Heath *et al.*, 2003); most late-onset cases are due to Gram-negative organisms, primarily selective subgroups of highly pathogenic *Escherichia coli* (Bonacorsi & Bingen, 2005). Similar frequencies for bacterial infections were also observed in a large Canadian study, with approx 50% of bacterial neonatal meningitis caused by group B streptococci, 25% *E. coli*, 8% other Gram-negative rods, 6% *L. monocytogenes*, and 3% non-typeable *Haemophilus influenzae* (Doctor *et al.*, 2001). Neonatal meningitis is associated with an overall mortality of approximately a quarter for both early and late cases. About a quarter of survivors also showed significant neurological sequelae (Hristeva *et al.*, 1993). Important predictors of adverse outcome are the presence and duration of seizures, coma, use of inotropes, and leukopenia (Klinger *et al.*, 2000), with the latter two reflecting the severity of septic shock.

Injury mechanisms

Although substantial neural damage is probably due to inherent tissue destruction in the directly infected brain, secondary mechanisms also play an important part. Many vertebrate neurons are known to commit suicide via apoptotic pathways after neurotropic viral infection. Such cell death might be an evolved strategy in multicellular organisms for limiting viral expansion (Allsopp & Fazakerley, 2000). Immature neurons are also more susceptible to infection-mediated cell death (Lewis *et al.*, 1999; Griffin, 2005). Damaged cells exude excitatory aminoacids (glutamate, aspartate) and reactive oxygen radicals that can lead to secondary damage in adjacent bystander cells (Folkerth, 2006; Matute *et al.*, 2006; Alvarez-Diaz *et al.*, 2007). Moreover, the massive release of intracellular contents in soluble form, particularly purinergic nucleotides, is a strong attracting stimulus for neighboring microglial cells (Nimmerjahn *et al.*, 2005; Haynes *et al.*, 2006; Koizumi *et al.*, 2007). In the presence of structural debris, these cells transform into phagocytes within hours (Streit *et al.*, 1988; Bohatschek *et al.*, 2001a; Raivich & Banati, 2004), and in the process contribute to heightened production of excitotoxins, inflammatory cytokines, and tissue-degrading enzymes that may augment neural damage (Taylor *et al.*, 2005; del Zoppo *et al.*, 2007; Makwana *et al.*, 2007).

One of the open questions in the neuropathology of early gestational infections is the reason for preferential damage to developing periventricular deep white matter; this pattern of injury, commonly observed in congenital rubella, cytomegalovirus, and early *T. gondii* infections, affects areas that are not particularly metabolically active. Compared with the densely packed cortical plate containing immature developing neurons, these areas are also relatively cell-poor (Rados *et al.*, 2006), as subcortical myelination only starts around birth (Holland *et al.*, 1986; Partridge *et al.*, 2004), and the large numbers of immature axonal fibers outweigh the early premyelinating oligodendrocyte precursors (DeSilva *et al.*, 2007). Although this location, with its complex arrangement of extracellular matrix embedding the axonal cables of the future white matter tracts (Rados *et al.*, 2006), may be particularly vulnerable due to a site-specific predilection of parasites and viruses, it is more likely that it reflects the effect of secondary damage due to the particularly high, local density of microglial phagocytes (Billiards *et al.*, 2006).

First described as the fountains of microglia in 1919 by Pio del Rio Hortega, these brain-resident, macrophage-related cells form well-characterized phagocytic clusters in the subependymal, embryonic, and fetal subcortical white matter, before they differentiate into the highly arborized cells characteristic of the late fetal, postnatal, and adult brain (Rio-Hortega, 1919, 1932; Rezaie & Male, 1999; Monier *et al.*, 2007). In addition to their periventricular location, these microglial clusters are also found at other border regions delineating gray and white matter interfaces (Rezaie *et al.*, 2005; Monier *et al.*, 2006), and appear to play a strategic role in pruning back exuberant axonal connections (Innocenti *et al.*, 1983; Billiards *et al.*, 2006). In the case of fulminant local infections, these "fountain of microglia" phagocytes may play an orchestrating role in severe and widespread axonal damage, followed by secondary gliosis.

In addition to direct microglial effects, viral nucleic acids have been recently shown to cause growth cone repulsion and collapse (Cameron *et al.*, 2007). In many cases this collapse is followed by neurite fragmentation, which could lead to additional activation of the neighboring microglial phagocytes (Hoopfer *et al.*, 2006; MacDonald *et al.*, 2006). Although the repulsive effect is mediated by toll-like receptors, it appears to be independent of nuclear factor (NF)-κB, which plays an otherwise pivotal role in the release of cytokine and reactive oxygen species in response to infection-associated stimuli (Cameron *et al.*, 2007). Growing axons appear particularly vulnerable to repulsive cues at turning points and at interfaces between different regions (Braisted *et al.*, 1999), via intraaxonal activation of calpain (Robles *et al.*, 2003; Zakharov *et al.*, 2005), which could lead to severe, synergistic damage to axons exiting or entering the embryonic and fetal cortical plate during the period of active viral infection.

On the whole, the proportion of cerebral palsy cases directly attributable to documented, perinatal, vertically acquired infections is estimated to be 5% or less in industrialized countries (Stanley *et al.*, 2000; Jacobsson & Hagberg, 2004). Nevertheless, this mode of causation is of clinical as well as molecular biological interest, because of: the ability to work with a clearly defined pathogenic process; the insights into structural basis of psychiatric disease; the role of brain-resident inflammatory cells in fetal axonal damage; and the ability to use the emerging potential of whole genome screening to uncover molecular pathways

involved in protecting or damaging the fetal brain during neuroinfectious disease.

Synergistic effects of systemic, extraneural infection, and hypoxia–ischemia

Exposure to infections strongly increases the risk of brain damage following hypoxic–ischemic events (Grether & Nelson, 1997). This increased risk is actually very similar to that in other neurological conditions. Prospective studies in Alzheimer patients have revealed prolonged impairment of cognitive function after the resolution of a systemic infection (Holmes *et al.*, 2003; Dunn *et al.*, 2005) and that this cognitive impairment is preceded by raised serum levels of interleukin 1β, probably produced by the resident microglial cells, during the process of infection (Holmes *et al.*, 2003). These relations were not confounded by the presence of any subsequent systemic infection or by baseline cognitive scores. In a separate study, patients with incident diagnosis of Alzheimer had a significantly, even though just moderately (+40%), higher incidence of systemic infections in the four years preceding the diagnosis (Dunn *et al.*, 2005). In the same line, augmented infection-associated risk has been shown in other neurodegenerative diseases such as spongiform encephalopathy (Allen & Cochran, 1977), Parkinson's disease (Liu *et al.*, 2003) and nonfamilial, sporadic amyotrophic lateral sclerosis (ALS; Swash & Schwartz, 1992; Cermelli *et al.*, 2003; Nguyen *et al.*, 2004). In particular, several studies have pointed to the importance of infection-related inflammation during early, including prenatal, life in setting the stage for the later onset of the progressive cell loss in midbrain dopaminergic neurons in this neurodegenerative disease (Mattock *et al.*, 1988; Martyn & Osmond, 1995; Carvey *et al.*, 2003).

Although traditionally, birth asphyxia has been considered the principal cause of cerebral palsy, epidemiological studies over the past 20–30 years have come to emphasize the importance of prenatal factors and suggest that severe intrapartum asphyxia plays a more minor role than previously supposed, accounting for approximately 10% of cases in term and near-preterm infants (Blair & Stanley, 2002). This perspective was partially challenged by a Swedish population-based report (Hagberg *et al.*, 2001). Using a series of indirect signs of birth asphyxia the authors stated that up to 30% of affected children might have experienced at least moderate deprivation of oxygen and nutrients, but that this is just one element of a multifactorial cause leading to poor motor and cognitive outcome (Jacobsson & Hagberg, 2004). However, there is a considerably higher risk, when both confounding causes – exposure to infection and prenatal or perinatal asphyxia, or other forms of a hypoxic–ischemic insult – come together. Nelson and Grether (1998) found that the presence of both greatly increased the risk of developing cerebral palsy (relative risk: 78) compared with either insult alone.

Pyrexia

Maternal pyrexia during labor has been related to an increased incidence of neonatal seizures and encephalopathy in the immediate postnatal period (Lieberman *et al.*, 2000; Impey *et al.*, 2001). It is unclear whether pyrexia is acting as a marker for underlying bacterial infection that triggers an inflammatory response that directly damages the brain or whether pyrexia makes the brain more vulnerable to the damaging effects of hypoxia–ischemia. Although both are possible, considerable data suggest that the latter is more probable: the combination of maternal pyrexia and fetal hypoxia–ischemia is associated with a higher (odds ratio 78, 95% confidence interval [CI] 4.8 to 406) risk of cerebral palsy than was observed with either factor alone, pointing toward a possible synergy between these two factors (Nelson & Grether, 1998); and animal studies describe clearly the direct correlation between increasing brain temperature and susceptibility to a variety of neurotoxic factors. Hypothermia can be neuroprotective after hypoxia–ischemia in neonatal animals, whereas hyperthermia increases brain injury after ischemia in adult rats (Dietrich *et al.*, 1990; Bona *et al.*, 1998). Maternal pyrexia, resulting from both microbial infection as well as noninfective causes such as epidural anesthesia, could therefore augment the deleterious effects of hypoxia on the fetal brain, possibly by increasing the cerebral metabolic rate and demand for oxygen.

Pro- and antiinflammatory cytokines

The molecular basis for the enhanced sensitivity to hypoxia–ischemia following inflammation mediated by bacterial products is the subject of intense clinical and basic research. Experimental studies using

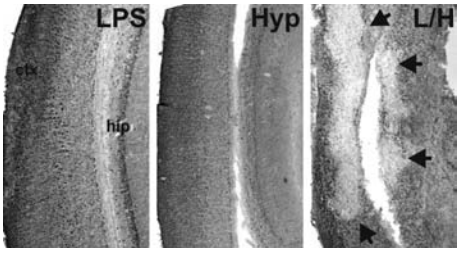

Fig. 16.1 The e ects of *Escherichia coli* lipopolysaccharide (LPS) 1 µg/g or hypoxia–ischemia (unilateral carotid occlusion followed by 8% oxygen for 30 minutes) either alone (LPS and Hyp) or in combination (with LPS administration preceding hypoxia–ischemia by four hours) on the brains of mouse pups at postnatal day 7. Neither insult in isolation causes significant cell death, indicated by Nissl staining. This is in contrast to the combined insult (L/H) which leads to loss of the greater part of the ipsilateral cortex. (From Kendall & Peebles, 2005.)

systemically applied bacterial endotoxin (lipopolysaccharide [LPS]) have shown a highly reproducible, synergistic effect in a variety of animal models including rats, mice (Fig. 16.1) and prehatching chicks (Lehnardt *et al.*, 2003; Yang *et al.*, 2004; Xue & del Bigio, 2005; Wang *et al.*, 2007). Very similar synergistic effects were also elicited with a much smaller, local injection of LPS into the cerebral ventricle, underscoring the intraneural site of the proinflammatory LPS activity (Coumans *et al.*, 2003).

Whether the effects of endotoxin are due to direct interference with energy metabolism is controversial. In glial/neuronal cultures, endotoxin has been shown to impair oxidative phosphorylation by inducing astrocytic nitric oxide release and reversible blockade of cytochrome oxidase (Mander *et al.*, 2005). However, in a recent study using combined LPS/hypoxia injury and continuous metabolite monitoring with magnetic resonance spectroscopy (MRS) in chick embryos in ovo, preexposure to endotoxin did not cause an earlier, more rapid, or stronger increase in cerebral lactate during the hypoxic insult (Wang *et al.*, 2008). Nonetheless, it did cause severe cerebral injury, suggesting that compromised energetics were unlikely to be an important etiological factor and that the effect was mediated by production of synergistic neurotoxic substances.

Molecular studies of the effects of LPS show they are transmitted through the classical endotoxin receptor toll-like receptor 4 on blood vessel endothelia and microglia (Lehnardt *et al.*, 2003; Kendall *et al.*, 2006; Gosselin & Rivest, 2008); downstream events include activation of the MyD88 and NF-κB components of the innate immunity cascade (Gosselin & Rivest, 2008), the release of prostaglandins (Olsson *et al.*, 2003) and inflammation-associated cytokines such as interleukin 1β (IL1β), IL6, and tumor necrosis factor α (TNFα). Moreover, maternal and/or fetal IL1β, IL6, and TNFα may play a critical dual role in initiating preterm labor as well as contributing to neonatal complications such as periventricular leukomalacia (Yoon *et al.*, 1996; Duggan *et al.*, 2001). Infection is not the only cause of increased levels of proinflammatory cytokines; hypoxia–ischaemia, trauma, and labor can all trigger a cytokine response (Savman *et al.*, 1998; Shalak *et al.*, 2002; Silveira & Procianoy, 2003), and it seems likely that a cytokine-mediated inflammatory response is the final common pathway leading to tissue damage after a variety of insults. Proinflammatory cytokines, including TNFα, IL1β, and interferon γ (IFNγ), have a variety of cerebral effects including a direct toxic effect on neurons and vulnerable oligodendrocyte precursor populations (Volpe, 2001; Taylor *et al.*, 2005), astrogliosis with release of nitric oxide and mitochondrial dysfunction (Bal-Price & Brown, 2001) as well as microglial activation with release of nitric oxide, superoxide, and a panel of other inflammation-associated molecules (Kloss *et al.*, 2001; Taylor *et al.*, 2005; Matute *et al.*, 2006; del Zoppo *et al.*, 2007).

Of potentially equal importance are a second group of cytokines including transforming growth factor β1 (TGFβ1) and IL10, with broadly antiinflammatory properties. TGFβ1 and IL10 are upregulated in most forms of CNS injury, including cerebral ischemia, excitotoxicity, trauma, and various types of neurodegenerative disease (Kiefer *et al.*, 1995; Dietrich *et al.*, 1999; Raivich *et al.*, 1999; Strle *et al.*, 2001). However, unlike the proinflammatory cytokines listed above, they are less upregulated following exposure to the inflammatory mediators of bacterial infection such as lipopolysaccharide, the cell wall breakdown product of Gram-negative bacteria (Xiao *et al.*, 1996). Both cytokines act as immunosuppressive factors that block the activation of lymphocytes, macrophages and related cell types such as brain microglia (Kloss *et al.*, 1997; Jones *et al.*, 1998; Strle *et al.*, 2001) as well as the microglial production of potentially neurotoxic molecules such as nitric oxide, superoxide, and IL1 (Vincent *et al.*, 1997; Terrone *et al.*, 2001; Robertson *et al.*, 2007). In the case of TGFβ1, the normally protective action of this cytokine is amply demonstrated in TGFβ1-null mice, which experience a persistent

neuroinflammatory condition, with excessive activation of microglia and astrocytes, interference with axonal transport, and considerably reduced neuronal survival following traumatic injury (Makwana *et al.*, 2007).

In vivo, application of TGFβ1 or IL10 strongly reduces the infarct size in cerebral ischemia in adult as well as in adolescent animals (Gross *et al.*, 1993; Spera *et al.*, 1998; Dietrich *et al.*, 1999). As proinflammatory cytokines mostly favor neuronal cell death while the antiinflammatory ones have a preventive effect, the balance between pro- and antiinflammatory cytokines in the neonatal brain is therefore likely to be critical in determining the initiation, development, and consequences of cerebral injury. There are several possible reasons why such variations in cytokine balance may occur: genetic polymorphisms may favor either a pro- or antiinflammatory response (Amory *et al.*, 2001); the type and severity of insult may influence the type of response, e.g., endotoxin is a potent stimulant of proinflammatory cytokines, but not TGFβ1; and following an inflammatory stimulus, the balance between pro- and antiinflammation may vary with time (Kiefer *et al.*, 1995; Khabar *et al.*, 1997). A two-phase inflammatory response has been described in neonates, with an antenatal wave of proinflammatory cytokines and a postnatal antiinflammatory response (Dammann *et al.*, 2001). These data suggest that in some neonates, levels of IL1, IL6, IL8, and TNFβ are high immediately following delivery and then fall to baseline levels by one week. Conversely, the antiinflammatory cytokine TGFβ1 increased continuously during the first week from low levels post delivery.

Are there protective effects of preconditioning?

Although synergistic effects have been demonstrated in numerous studies, in different species and experimental models there appears to be a narrow time window, in terms of the interval between exposure to ligand that stimulates innate immunity and subsequent hypoxia, in which sensitization and enhanced neurodestructive action occur (see, however, Wang *et al.*, 2007). In neonatal mice exposed to hypoxic–ischemic insult and LPS, most synergy was observed when LPS application preceded the hypoxic–ischemic insult by 12 hours, disappearing with coapplication of LPS and hypoxic–ischemia, or with LPS preceding the hypoxic–ischemia by 24 hours or more (Kendall *et al.*, 2006). In fact, longer gaps between a one-time application of endotoxin and standard hypoxic–ischemic damage can even elicit a paradoxical effect, causing reduction in the volume of infarct via protective preconditioning (Ahmed *et al.*, 2000).

On the molecular level, this inflammatory preconditioning is mediated, at least in the adult, via a number of different molecular pathways involving toll-like receptors 4 and 9 (Stevens *et al.*, 2008), superoxide, nitric oxide and cyclic GMP (Kawano *et al.*, 2007; Orio *et al.*, 2007), and TNFα (Rosenzweig *et al.*, 2007). Nevertheless, it is doubtful if this protective effect is maintained during continuous exposure to bacterial products. In fact, ongoing repetitive exposure seems to have a much more detrimental effect (Wang *et al.*, 1999; Bohatschek *et al.*, 2001b; Stolp *et al.*, 2007), and it is this ongoing continuous exposure that is probably best attuned to mirror the results of infection on coincident brain damage.

Primary exposure to systemic infection alone

Perinatal infection is a major confounding risk factor in spastic cerebral palsy (CP) and cystic periventricular leukomalacia (cPVL). By itself, the presence of perinatal infection appears to contribute to approximately 10% of the CP cases in term babies, and up to 30% in preterm babies according to Grether and Nelson (1997). Use of antibiotics during pregnancy and maternal fever also show significant correlation with CP (Lieberman *et al.*, 2000; Impey *et al.*, 2001; Jacobsson *et al.*, 2002). Although other causes of maternal fever are possible, it may also simply reflect the presence of previous or ongoing infection. At the moment there is still a debate about whether perinatal infection has a stronger effect in term, preterm or very premature babies (Nelson & Grether, 1998; Jacobsson *et al.*, 2002). However, there is a general consensus that infection raises the overall risk. Thus, a metaanalysis of 30 studies linking clinical and histological chorioamnionitis, CP, and cPVL showed, in both cases, a statistically significant, twofold elevated CP risk in preterm and a 4.7-fold increase in term neonates (Wu & Colford, 2000). Similar infection-mediated risks were also observed for cPVL. Recent data from the ORACLE trial have highlighted again the possible role of bacterial infection as the cause of both chorioamnionitis and

perinatal brain injury; erythromycin use in women in symptomatic preterm labor with intact membranes was associated with an increase in functional impairment in their children (mainly mild), and cerebral palsy was more common when the mother had received either erythromycin or co-amoxiclav, with the greatest risk observed in those who had received both antibiotics together (Kenyon *et al.*, 2008). These data suggest that although maternal antibiotic treatment might clear or suppress bacterial colonization it will not necessarily have an antiinflammatory effect; in fact, it is possible that it could make matters worse, either by stimulating toll receptors through release of immunogenic bacterial cell wall products and DNA, or by prolonging pregnancy and therefore fetal exposure to inflammatory molecules.

Mechanistically, a key question is whether or to what extent these effects are: (i) due to the presence of infectious stimulus alone; (ii) due to the synergy with independent, birth-associated asphyxia, discussed in the preceding paragraph; or (iii) caused by an additional hypoxic–ischemic event brought on by maternal, fetal, or neonatal infection. This additional hypoxic–ischemic event could be caused by intracerebral hemorrhage, thromboembolism or reduction in placental perfusion, itself brought on by infection in the maternofetal unit. While the effect of (ii), the synergy between inflammation and perinatal asphyxia, is now well documented, there is still ongoing debate on the relative impact of (i) and (iii), i.e., whether extraneural infection alone is sufficient to elicit brain pathology.

In favor of the infection-alone hypothesis, experimental models clearly show that maternal or fetal infection can produce typical brain pathology and neurodevelopmental abnormalities in the offspring (Patterson, 2007). In rodents, maternal respiratory infection during mid-gestation was associated with widening of cerebral ventricles, subcortical white matter abnormalities, and atypical behavior with social interaction deficits and prepulse inhibition, consistent with those seen in schizophrenia and autism (Fatemi *et al.*, 2002; Shi *et al.*, 2003). Similar effects are also elicited by injection of double-stranded RNA into the mother, evoking an antiviral inflammatory response (Zuckerman *et al.*, 2003). At the molecular level, blocking IL6 or increasing IL10 strongly reduces fetal brain damage (Smith *et al.*, 2007; Meyer *et al.*, 2008), identifying some of the key cytokines involved in this virus-mediated maternofetal immune response. Similar findings, with ventricle expansion and reduction of rat or mouse subcortical white matter were also observed following maternal injection of LPS, an immunogenic bacterial component (Robertson *et al.*, 2006).

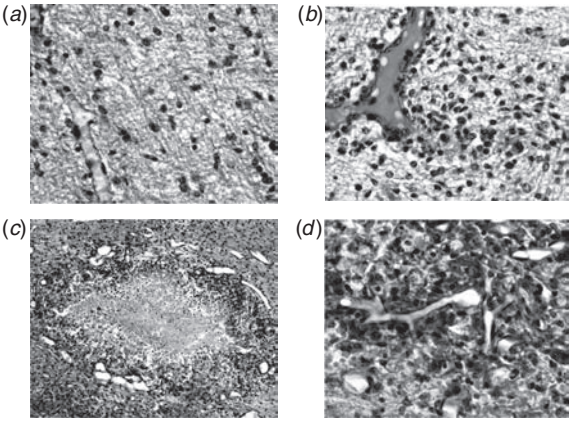

Fig. 16.2 White matter changes in ovine fetal brain 72 hours following systemic fetal injection of 100 ng/kg *Escherichia coli* lipopolysaccharide at 0.65 gestation. (*a*) Normal white matter for comparison (hemotoxylin and eosin, magnification ×400). (*b*) New vessel formation and gliosis in the white matter (hemotoxylin and eosin, magnification ×400). (*c*) Cystic lesion in white matter (hemotoxylin and eosin, magnification ×100). (*d*) New vessels and gliosis at the edge of the cyst shown in (*c*) (hemotoxylin and eosin, magnification ×400). (From Peebles *et al.*, 2003b.)

In the same vein, application of LPS alone to mid-gestation fetal sheep results in an even more pronounced white matter injury typical of cPVL. These changes, shown in a number of studies from groups in the UK, Australia, Sweden, and Netherlands, include focal inflammatory infiltrates, cystic lesions, and intraparenchymal hemorrhage (Fig. 16.2), and were accompanied by microglia activation in the white matter, astrocyte damage, and loss of oligodendrocytes (Duncan *et al.*, 2002; Mallard *et al.*, 2003; Peebles *et al.*, 2003; Garnier *et al.*, 2006). Why fetal sheep are more susceptible than rodents and whether sheep or rodents are more representative of human babies at risk, remain unclear. However, the fetal sheep model definitely carries the potential to identify the physiological and molecular pathogenicity. The fact that chronic, continuous application of very-low-dose LPS over several days is not associated with a drop in blood pressure and oxygen saturation but still causes considerable diffuse axonal damage (Duncan *et al.*, 2006) appears to underscore the presence of infection/inflammation-only mechanisms at least in some fetal white matter damage models.

This does not exclude the involvement of additional hypoxic–ischemic factors under the everyday clinical or experimental conditions. Occlusion of uterine or umbilical arteries in the fetal sheep is known to result in focal subcortical white matter damage (Mallard *et al.*, 2003; Duncan *et al.*, 2004). Moreover, single bolus injections of endotoxin cause a significant reduction in placental function, fetal blood oxygen saturation, and blood pressure (Peebles *et al.*, 2003), and much more pronounced focal pathology, suggesting a very clear additional, hypoxic component in the watershed perfusion areas of the deep periventricular and subcortical white matter.

On the vascular side, endotoxin and other breakdown products of pathogens clearly affect the normal function of endothelial cells in the brain. Elicited changes include strong upregulation of vascular cell adhesion molecules (ICAM1, VCAM1, selectins), enhanced synthesis of basal membrane-degrading enzymes, transmigration of neutrophils and other circulating leukocytes, and an overall reduction in the integrity of the blood–brain barrier (Kim & Koh, 2000; Bohatschek *et al.*, 2001b; Cunningham *et al.*, 2005; Rosenberg *et al.*, 2007), increasing vessel fragility and the probability of thromboembolic and hemorrhagic complications. Preterm infants whose amnion is acutely inflamed are at a three- to fourfold greater risk of developing intraventricular hemorrhage (Yoon *et al.*, 1995, 1996; Salafia *et al.*, 1995). Moreover, application of antimicrobial therapy after premature rupture of membranes has been associated with a clearly reduced risk (Mercer & Arheart, 1995; Egarter *et al.*, 1996).

Infectious agents are known to be linked to increased risk of coronary heart disease and, by extension, to other related cardiovascular conditions such as stroke. Part of the enhanced risk is due to the thrombo-ischemic complications associated with bacteremic conditions (Valtonen *et al.*, 1993). Implicated pathogens include *Chlamydia pneumoniae*, *Helicobacter pylori*, CMV, and herpesviruses, as well as periodontal infections (Armitage, 2000; Seymour *et al.*, 2007), even though evidence supporting a causative role of chronic infections in coronary heart disease is still largely circumstantial (Solenski, 2007). For example, short-term use of different groups of antibiotics, including macrolides, quinolones, tetracyclines, cephalosporins, penicillins or trimethoprim-sulfamethoxazoles, does not reduce the risk of first-time stroke in the over-65-years population (Luchsinger *et al.*, 2001). However, in young children with arterial ischemic stroke (AIS), there is a threefold increase in preceding varicella infection compared with published population rates, and varicella-associated AIS accounts for nearly a third of childhood AIS (Askalan *et al.*, 2001). Similarly, *C. pneumoniae* and periodontal infection appear to fulfill the criteria of independent predictors of higher risk in stroke (Sacco, 2001). Importantly, the same mechanisms also appear to operate much earlier in life, in prenatal and perinatal brain, underscoring a shared predilection for infection-mediated thromboembolic events.

It is clear that there is a spectrum of involvement of infection in perinatal brain injury, ranging from cases that are solely related to infection and those where infection plays no role, but factors such as hypoxia–ischemia are most important; there is also a middle ground, probably in substantial numbers of cases, where infection-mediated inflammation and hypoxia–ischemia occur at the same time. Data, mainly from animal studies, suggest that when this occurs there is the potential for synergistic interaction with worse outcome than observed with either factor alone; however, further clinical studies are needed to confirm this observation. However, it is likely that in many cases fetal hypoxia could be induced by infection leading to increased production of cytokines and upregulation of adhesion factors, further leading to both placental hypoperfusion and intracerebral events such as hemorrhage and thromboembolism. It is clear that understanding the mechanisms behind infection-induced toxicity and the synergy between infection and hypoxic–ischemic insult, and the resulting damage to the immature brain, clearly hold the promise of reducing, and possibly preventing, neurological disability in children at risk.

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Chapter 17

Hypoxic–ischemic encephalopathy

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The role of hypoxia–ischemia in perinatal brain injury

Injury to the brain depends on not only the type and severity of insult, but also the maturity of the tissue. Hypoxic–ischemic encephalopathy is generally considered to be characteristic of the term infant who has experienced a severe perinatal deficit in cerebral oxygen delivery leading to disruption of cerebral energy metabolism (Volpe, 1994). This is frequently followed by a global hypoxic–ischemic injury, with a widespread although not uniform distribution of apoptotic and necrotic cell death. Nevertheless, focal cerebral infarction is also seen in term infants, and may be underdiagnosed unless sophisticated techniques such as diffusion-weighted magnetic resonance imaging (MRI) are used (Cowan *et al.*, 1994). Hypoxic–ischemic changes are also seen in many stillbirths although in these infants apoptotic death may be particularly prominent (Edwards *et al.*, 1997).

Uncertainty about the role of intrauterine hypoxemia or cerebral ischemia is exacerbated by the imprecise measures of fetal oxygenation or cerebral blood flow available to clinicians. Observations of clinical variables such as cardiotocography or meconium staining of the liquor may mislead if interpreted as precise measures of fetal cerebral hypoxia and ischemia (Nelson *et al.*, 1998). However, more accurate techniques such as magnetic resonance spectroscopy (MRS) have defined at least a subgroup of infants with characteristic hypoxic–ischemic injury, and it is clear that cerebral hypoxia and/or ischemia is involved in a significant proportion of neonatal encephalopathy (Azzopardi *et al.*, 1989).

However, basic research into the mechanisms of cerebral injury is beginning to suggest that the apparent distinction between hypoxic–ischemic and other forms of cerebral damage may be less clear than has previously been thought. It is now apparent that not only do infection and sepsis often cause cerebral hypoxia, but that hypoxia–ischemia triggers inflammatory cascades within the brain (Grether & Nelson, 1997; Hagberg & Mallard, 2005). Different pathologies can activate common cell death pathways. This chapter will focus on these processes in the developing brain, examining first the pathophysiology of brain damage, then discussing mechanisms of cell injury.

The pathophysiology of hypoxic–ischemic cerebral injury

Initial and delayed injury after perinatal hypoxia–ischemia

MRS permits noninvasive observation of intracellular pH (pHi), and the cerebral concentrations of adenosine triphosphate (ATP), phosphocreatine (PCr), inorganic phosphate (Pi), and lactate. When ATP generation is impaired energy flux is maintained by the breakdown of PCr while Pi increases, so that a decline in the ratio [PCr]/[Pi] is a valuable indicator of impaired energy metabolism, even in the presence of normal or near-normal concentrations of ATP. Impaired oxidative phosphorylation is also associated with increased intracerebral lactate (Cady, 1990).

Infants with hypoxic–ischemic encephalopathy show characteristic abnormalities in cerebral energy metabolism, which is frequently normal soon after birth, but shows a progressive decline in [PCr]/[Pi] and increase in lactate some hours later. Infants displaying this phenomenon develop severe neurodevelopmental impairment or die and there is a close relationship between the magnitude of the late decline in [PCr]/[Pi], reduced brain growth, and the severity

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of neurodevelopmental impairment one and four years later (Roth *et al.*, 1997; Hanrahan *et al.*, 1999).

These findings suggested the concept of "secondary energy failure," which has been developed and extended by several groups (Hope *et al.*, 1984; Hanrahan *et al.*, 1996; Martin *et al.*, 1996). Delayed declines in [PCr]/[Pi] beginning some 8–12 hours after birth asphyxia have been confirmed and quantitated in a number of studies. Interestingly it was found that delayed disruption of cerebral energy metabolism is associated with a normal or increased in pHi, in clear contrast to acute hypoxia–ischemia when pHi characteristically falls, and increased pHi is a strong predictor of a poor neurological outcome.

The above described data have led to the hypothesis that hypoxic–ischemic injury occurs in at least two main phases, with a primary defect in cerebral energy production during hypoxia–ischemia precipitating the later events. This has been supported by studies of global hypoxia–ischemia in the newborn piglet (Lorek *et al.*, 1994), and in focal stroke in rat pups (Palmer *et al.*, 1990; Blumberg *et al.*, 1996). During hypoxia–ischemia intracerebral [PCr]/[Pi] and pHi fall, and lactate increases. Eventually [ATP] declines, but even if this transiently falls to undetectable levels the reprovision of substrate usually causes the metabolites to return to normal values within an hour or two. Some hours later [PCr]/[Pi] again declines (Fig. 17.1), lactate increases, but pHi becomes alkaline. There is a dose–response relationship between the severity of the hypoxic–ischemic insult and the magnitude of the secondary changes in cerebral energy metabolism (Lorek *et al.*, 1994). Equally, the more severe the cerebral metabolic impairment, the more extensive the histological injury (Blumberg *et al.*, 1996; Gilland *et al.*, 1998a).

However, although it has been useful, the concept of biphasic cerebral damage is an oversimplification. Diffusion-weighted MRI studies in piglets have shown that although changes in the apparent diffusion coefficient of brain (an index of the restriction of movement of water, and thus of cell membrane integrity) closely parallel changes in [PCr]/[Pi] during secondary energy failure, the changes are not uniform through the brain. Rather, abnormal diffusion frequently begins laterally and then progresses towards the parasagittal and central regions (Thornton *et al.*, 1998). Clearly a change in global [PCr]/[Pi] obscures considerable complexity of regional response.

MRI of infants who develop secondary energy failure after birth asphyxia confirms that damage is distributed heterogeneously throughout the brain although injury to the basal ganglia and deep structures of the brain seem to predict adverse neurological outcome most accurately (Rutherford *et al.*, 1998) (Fig. 17.2). Localized MRS measurements also show that metabolite concentration abnormalities differ in different regions of the brain; for example, lactate

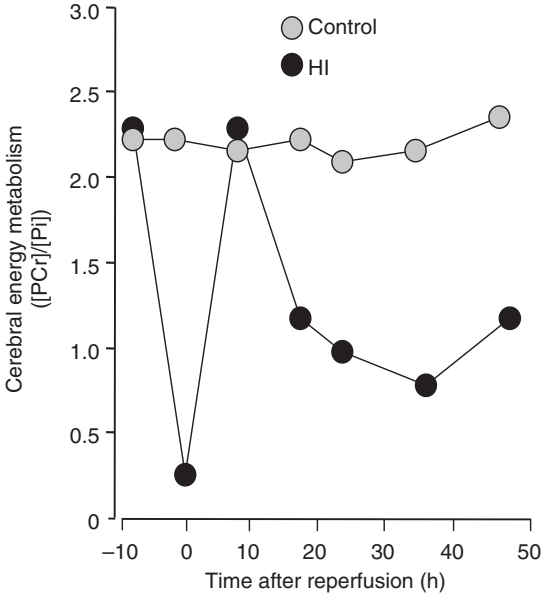

Fig. 17.1 Secondary impairment of energy metabolism following hypoxia–ischemia in the neonatal rat. Cerebral energy metabolism was measured by magnetic resonance spectroscopy in control animals and those after hypoxia–ischemia (HI). Following reperfusion, energy metabolism recovers but declines again after 10 hours. (Adapted from Blumberg *et al.*, 1996.)

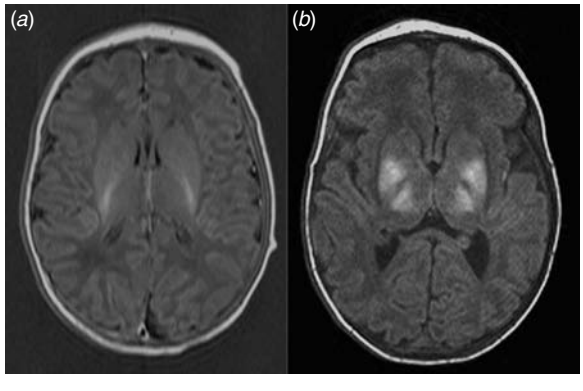

Fig. 17.2 (*a*) T1-weighted magnetic resonance image from a normal newborn infant, showing characteristic high signal in the posterior limb of the internal capsule, and (*b*) image of an infant with severe perinatal hypoxic–ischemic injury showing high signal in the basal ganglia and thalamus, and loss of signal in the posterior limb of the internal capsule.

seems to be higher in the thalamus than the occipital region (Penrice *et al.*, 1996).

It has been known for many years that some regions of the brain are constitutively susceptible to hypoxic–ischemic injury, while others are relatively resistant. However, the situation is particularly complex because regions distant from, but in synaptic contact with, a site of damage may be injured after the insult (diaschisis) due to the interruption of neuronal circuitry leading to ante- and retrograde axonal degeneration and lack of trophic influences needed to maintain cellular integrity (Joashi *et al.*, 1999; Taylor *et al.*, 2006).

Abnormal cerebral energy metabolism also continues for much longer than was previously thought. In children who are neurologically normal one year following birth asphyxia, cerebral lactate concentrations rapidly fall. However, in those who develop neurodevelopmental impairment lactate can be detected in the brain for many months (Hanrahan *et al.*, 1998), and is associated with a marked intracellular alkalosis (Robertson *et al.*, 1999). Similar prolongation of abnormal cerebral metabolism has been noted after adult stroke. The mechanism is unknown, but in adult rats moderate hypoxia–ischemia is followed by a very prolonged period of increased apoptotic death in affected areas (Du *et al.*, 1996) and injury after hypoxia–ischemia in neonatal rats sometimes develops with a considerable delay (Geddes *et al.*, 2001). These findings emphasize that injury develops for an extended period after hypoxia–ischemia.

Cellular and molecular mechanisms of hypoxic–ischemic brain injury

The deficit in energy charge induced by hypoxia–ischemia leads to a primary failure to maintain transmembrane ionic gradients, release of neuroactive compounds into the extracellular compartments and the activation of a series of mechanisms that if sustained will lead to immediate cell death. If the individual is resuscitated, these acute alterations are completely or partly reversed but the complex process has been started in which multiple interrelated factors may produce secondary brain injury.

The precise mechanisms of damage are incompletely understood but some components of the process have been elucidated. Excitatory amino acids (EAA), mitochondrial impairment, intracellular calcium regulation, oxygen free radical (OFR) generation including nitric oxide, apoptotic mechanisms, changes in the availability of trophic factors and the immunoinflammatory system are all implicated in the process, as are other factors (Fig. 17.3). Interestingly, the entire process is extremely temperature dependent, and a reduction in brain temperature of only 3–4 °C after hypoxia–ischemia greatly reduces the amount of cell death, offering a potentially important method of neuroprotective therapy (Thoresen & Wyatt, 1997; Edwards *et al.*, 1998; Taylor *et al.*, 2002; Gluckman *et al.*, 2005; Shankaran *et al.*, 2005).

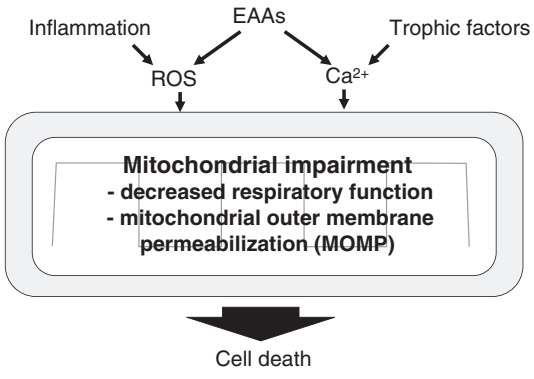

Fig. 17.3 Mitochondria play a central role in cell death. Multiple stimuli such as free radicals, excitatory amino acids, or adenosine triphosphate (ATP) depletion lead to mitochondrial dysfunction. Severe disruption leads to failure of ATP generation and if this cannot be compensated from other sources such as PCr it will lead to rapid necrosis. Less severe damage may induce the secretion of proapoptotic factors into the cytosol and activation of apoptotic signaling pathways and apoptotic execution. EAA, excitatory amino acid; ROS, reactive oxygen species.

Excitatory amino acids

Glutamate and aspartate are the main excitatory transmitters in the brain, but they have been known for a long time to exert toxic effects (excitotoxicity) if applied in excess to the nervous system (Lucas & Newhouse, 1957; Olney & Ho, 1970). Both *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)/kainate receptors are expressed on neurons and oligodendroglial precursors (preferentially on somata) in vulnerable areas of gray and white matter (Follett *et al.*, 2004; Johnston, 2005; Salter & Fern, 2005). The expression of EAA receptors is upregulated in the immature human brain which reflects the critical role of these receptors for brain development (McDonald & Johnston, 1990). Hence, the immature brain is also more vulnerable to excitotoxicity (especially NMDA) than the adult (McDonald *et al.*, 1988).

There is considerable evidence for a role of EAAs in the process leading to hypoxic–ischemic brain injury. Extracellular concentrations of EAAs and to some extent glycine increase in gray matter during neonatal hypoxia–ischemia (Hagberg *et al.*, 1987) followed by a secondary increase during reflow (Tan *et al.*, 1996; Puka-Sundvall *et al.*, 2000; Thoresen *et al.*, 1997), and a glutamate depletion has been detected from axon and oligodendroglial precursors (Back *et al.*, 2006). Blocking NMDA receptors before or after hypoxia–ischemia reduces subsequent neuronal damage (McDonald *et al.*, 1987; Hagberg *et al.*, 1994) and during "in vitro ischemia," NMDA receptor activation results in Ca^{2+}-dependent injury of oligodendroglial processes (Salter & Fern, 2005). AMPA blockade reduces gray and white matter damage when given after hypoxia–ischemia (Hagberg *et al.*, 1994; Follett *et al.*, 2004). The mechanism of excitotoxicity in response to hypoxia–ischemia probably involves perturbation of Ca^{2+} homeostasis, triggering nitric oxide and reactive oxygen species (ROS) production with subsequent mitochondrial impairment and activation of lipases and proteases. Some NMDA receptor *antagonists* can, however, induce apoptosis in the immature brain (Ikonomidou *et al.*, 1999) which would complicate the use of such drugs for cerebroprotection.

Calcium homeostasis

Calcium (Ca^{2+}) ions are ubiquitous intracellular second messengers, acting as key regulators of numerous cellular functions (Miller, 1987). In order to allow e cient Ca^{2+}-dependent signaling, the intracellular Ca^{2+} concentration (Ca^{2+}ic) is strictly regulated at a low level of 100 nM, i.e., 10 000 times lower than the extracellular concentration (Miller, 1991). The large electrochemical gradient is being upheld through ATP-requiring processes at the level of the cell membrane (Na^+/Ca^{2+} exchange and Na^+/K^+-ATPase, Ca^{2+}-ATPase), mitochondria and endoplasmic reticulum (Miller, 1991). In the adult brain, transmembrane ionic gradients cannot be maintained and a rapid depolarization occurs after a few minutes of complete anoxia or ischemia with a concomitant rise of Ca^{2+}ic (Siesjö, 1986; Silver & Erecinska, 1990). A marked rise of Ca^{2+}ic may trigger a number of toxic processes such as activation of calpains, OFR, apoptosis, phospholipases, endonucleases, and nitric oxide production.

Intracellular calcium regulation is thus considered to play an important role in the cell's response to injury (Choi, 1995). However, its significance in immature brain injury is less clear. We presently do not know to what extent intracellular calcium increases during hypoxia–ischemia. In studies in vitro, the rise of Ca^{2+}ic tends to be slower and less pronounced in immature neurons (Bickler *et al.*, 1993; Bickler & Hansen, 1998). However, Ca^{2+} accumulates to some extent in the brain tissue during hypoxia–ischemia (Stein & Vannucci, 1988) and calcium-dependent enzymes such as calpains and phospholipase C are activated (Chen *et al.*, 1988; Blomgren *et al.*, 1995a), which offers some indirect information in support of an increase of Ca^{2+}ic in the immature brain during hypoxia–ischemia.

It is equally unclear exactly what happens to Ca^{2+}ic after delayed injury. In the 5–72 hours following a period of hypoxia–ischemia there is a delayed accumulation of calcium in regions with brain injury (Stein & Vannucci, 1988) (see also below "Mitochondrial impairment"). In stroke models in adult rodents, there are waves of NMDA-receptor-dependent depolarizations (spreading depression) in border-zone areas accompanied by neuronal uptake of Ca^{2+}. In the immature brain, spreading depression occurs at a slower rate and the degree of Ca^{2+} influx appears to be lower than in the adult counterpart (Takita *et al.*, 2004). The pathophysiological role of these Ca^{2+} transients is yet uncertain but could theoretically contribute to NMDA-receptor-dependent excitoxicity during the reperfusion phase. The calcium-entry blocker flunarizine administered prior to (but not after) hypoxia–ischemia attenuated brain injury in 7-day-old rats (Silverstein *et al.*, 1986; Chumas *et al.*, 1993) but high doses were administered and nonselective effects of the drug make the interpretation di cult.

Mitochondrial impairment

Morphology

There seems to be a shift towards a more juxtanuclear mitochondrial localization after hypoxia–ischemia (Puka-Sundvall *et al.*, 2000; Northington *et al.*, 2001). Application of electron microscopy combined with the oxalate–pyroantimonate technique revealed a progressive accumulation of calcium in the endoplasmic reticulum, cytoplasm, nucleus, and, most markedly, in the mitochondrial matrix of neurons 30 minutes to three hours after hypoxia–ischemia (Puka-Sundvall *et al.*, 2000). Some mitochondria developed a considerable degree of swelling reaching a diameter of several

micrometers at three hours of reflow, whereas the majority of mitochondria appeared moderately affected. Chromatin condensation was observed in the nuclei of many cells with severely swollen mitochondria with calcium deposits.

Mitochondrial function

During early recovery after hypoxia–ischemia, high-energy phosphates in the cerebral cortex are restored as previously mentioned (Blumberg *et al.*, 1996; Gilland *et al.*, 1998a). During this phase, the 2-deoxyglucose (2-DG) utilization was increased, which correlated with increased levels of tissue lactate (Gilland & Hagberg, 1996) and a depression of mitochondrial respiration (Gilland *et al.*, 1998a). We have also found that post-hypoxic–ischemic administration of an NMDA receptor antagonist normalized 2-DG utilization, lactate levels, improved mitochondrial respiration, and attenuated cortical brain injury (Gilland & Hagberg 1996; Gilland *et al.*, 1998a,b). These data suggest that NMDA receptor activation in the early recovery phase depresses mitochondrial respiration with a compensatory increase of anaerobic glucose cycling to lactate, which precedes development of cortical brain injury. Interestingly, a similar pattern of increased glucose use occurred in the central nervous system (CNS) of asphyxiated infants, particularly in brain regions that were subsequently injured (Blennow *et al.*, 1995). Such an increase in glucose utilization occurred in parallel with marked elevations of glutamate in the cerebrospinal fluid (Hagberg *et al.*, 1993), implying that hypoxic–ischemic brain injury also in postasphyxiated infants is preceded by a phase of mitochondrial impairment related to activation of EAA receptors.

Apoptotic mechanisms

Apoptosis in the immature brain

Cell death is often classified as apoptotic or necrotic based on biochemical or morphological criteria (Martin *et al.*, 1998). Necrotic cell death is triggered by an overwhelming insult resulting in complete loss of membrane integrity and leaking of cytoplasmic contents into the extracellular matrix related to loss of energy. Apoptotic cells do not lose membrane integrity and the organelles remain largely intact until the final stages when cell fragments bud off as apoptotic bodies that are subsequently phagocytosed by microglia or healthy neighboring cells.

Apoptosis was initially recognized for its role in development. In some brain regions, half of the neurons die by apoptosis during normal brain development (Raff *et al.*, 1993). Therefore it is entirely appropriate that many apoptosis-related factors are upregulated in the immature brain, such as caspase 3, Apaf-1, Bcl-2, and Bax (Merry *et al.*, 1994; Blomgren *et al.*, 2001; Ota *et al.*, 2002). Multiple apoptotic pathways converge on caspase 3, so this protease is critical in the execution of neuronal apoptosis both during brain development and after acute injury (Kuida *et al.*, 1996). Caspase 3 appears to be particularly important in the brain, because mice devoid of caspase 3 through genetic targeting displayed a hyperplastic, disorganized brain, whereas other organs appeared normal (Kuida *et al.*, 1996). The constitutive levels of caspase 3 and the activation after injury are severalfold more pronounced in the immature brain (Hu *et al.*, 2000). Release of cytochrome C from mitochondria, leading to activation of caspase 9 and subsequently caspase 3, is also more pronounced in the immature brain compared with the juvenile and adult brain (Zhu *et al.*, 2005). In summary, due to ongoing apoptotic processes during brain development it appears that the apoptotic biochemical machinery is highly upregulated in the immature brain, which might confer heightened vulnerability.

Caspase-dependent cell death and apoptosis-inducing factor in hypoxia–ischemia

Studies suggest that mitochondria regulate apoptotic cell death through their capacity to undergo mitochondrial outer membrane permeabilization (MOMP) and release of proapoptotic proteins (Green & Kroemer, 2004) (Fig. 17.3). Cytochrome C (Cyt C), and other apoptogenic proteins, such as apoptosis-inducing factor (AIF), endonuclease G, SMAC/Diablo, and HtrA2/Omi, are released from the mitochondrial intermembrane space. Bax, Bad, Bid, and other members of the Bcl-2 family are involved in the regulation of mitochondrial release of proapoptotic proteins. Cyt C interacts with APAF-1, ADP, and pro-caspase 9 to form the heptameric apoptosome, leading to activation of caspase 9, which in turn cleaves and activates pro-caspase 3 (Green & Kroemer, 2004). AIF, on the other hand, promotes apoptosis in a caspase-independent manner (Susin *et al.*, 1999). In addition, the downstream activation of executioner caspases such as caspase 3 can be triggered through Fas receptor-mediated activation of

caspase 8 without involvement of mitochondria, the so-called extrinsic pathway.

Apoptotic cells have been reported following hypoxic–ischemic injury in the immature brain (Mehmet *et al.*, 1994) and the amount of apoptosis is proportional to the severity of the hypoxic–ischemic insult. Apoptosis is also found in the brains of infants who die after intrauterine insults or perinatal hypoxia–ischemia (Edwards *et al.*, 1997; Scott & Hegyi, 1997). Most morphological studies suggest, however, that mixed necrotic-apoptotic phenotypes predominate after immature brain injury (Portera-Cailliau *et al.*, 1997) but ample evidence supports the concept that apoptotic mechanisms are critically involved (Fig. 17.3) (Blomgren & Hagberg, 2006).

The mechanism behind the appearances of mixed necrotic-apoptotic forms of death appears to lie in the availability or otherwise of energy to the cell. Apoptosis requires energy to complete (Leist *et al.*, 1997) and if a cell's energy supply fails, due for example to disruption to blood flow caused by damage to the local vascular system, the cell will be unable to complete the apoptotic program but instead will die by necrosis. Recent work has shown that following hypoxia–ischemia in the immature rat brain there is formation of a functional apoptosome, simultaneously with loss of structurally intact and functioning mitochondria. This may cause incomplete packaging of nuclear and cytoplasmic contents and a hybrid of necrotic and apoptotic features (Northington *et al.*, 2007).

Caspase 3 is markedly activated after hypoxia–ischemia in the immature brain (Cheng *et al.*, 1998; Zhu *et al.*, 2000; Wang *et al.*, 2001) and cells with the cleaved active form of caspase 3 colocalize with markers of DNA fragmentation in injured brain regions (Zhu *et al.*, 2000). Caspase 3 inhibitors (Cheng *et al.*, 1998) as well as transgenic overexpression of X-linked inhibitor of apoptosis (XIAP) (Wang *et al.*, 2004) attenuate caspase 3 activation and provide a considerable degree of neuroprotection in the neonatal setting in some (Cheng *et al.*, 1998) but not in all studies (Zhu *et al.*, 2003; Joly *et al.*, 2004).

There are data to suggest that the extrinsic pathway is activated in response to hypoxia–ischemia (Felderhoff-Mueser *et al.*, 2000) and Fas receptor deficiency seems to confer some degree of protection in neonatal hypoxia–ischemia (Graham *et al.*, 2004). Most data, however, suggest that activation of the intrinsic pathway is the key event in the immature brain. Assembly of the apoptosome is easily induced in homogenates from the immature brain (Gill *et al.*, 2002), Cyt C is released to the cytosol in response to hypoxia–ischemia (Northington *et al.*, 2001; Zhu *et al.*, 2003) and caspase 9 is activated (Hallin *et al.*, 2006). In addition, other proapoptotic proteins such as AIF (Zhu *et al.*, 2003), SMAC/Diablo (Wang *et al.*, 2004), and HtrA2/Omi (Wang *et al.*, 2004) translocate from the mitochondria to a nuclear localization, suggesting that proapoptotic proteins are indeed released during the early recovery phase after hypoxia–ischemia (Fig. 17.4). We find that cells with immunohistochemical translocation of Cyt C and AIF often exhibit signs of DNA fragmentation and nuclear condensation, and these cells are preferentially localized in regions with early loss of the neuronal marker microtubule associated protein-2 (MAP-2) (Zhu *et al.*, 2000, 2003). These data suggest an association between mitochondrial release of proapoptotic proteins and brain injury, but its direct role in the process leading to cell death is incompletely understood.

Recently, we found that development of brain injury in immature mice also depends on AIF (Zhu *et al.*, 2007a). The distribution of AIF translocation matches the accumulation of poly(ADP-ribose) (PAR) suggesting that activation of poly(ADP-ribose) polymerase (PARP) might trigger AIF release from mitochondria (Hagberg *et al.*, 2004). AIF is released to the cytosol, binds to another protein, cyclophilin A (CyA) (Zhu *et al.*, 2007b) and the AIF–CyA complex translocates to the nucleus and triggers DNA degradation. The release of proapoptotic proteins from mitochondria depends on induction of MOMP (Fig. 17.4). It remains unclear how MOMP is triggered in the immature brain after hypoxia–ischemia but does not seem, as in adults, to be mediated by opening of a cyclophilin-D-dependent mitochondrial permeability transition pore (Puka-Sundvall *et al.*, 2001).

Involvement of Bcl-2 family proteins

Transgenic mice overexpressing human Bcl-xL postnatally were resistant to neonatal hypoxia–ischemia (Parsadanian *et al.*, 1998). In addition, hypoxia–ischemia induced an increase in Bax in mitochondrial-enriched cell fractions, which occurred in parallel with an increase of Cyt C in the cytosol preceding activation of caspase 3 in the neonatal thalamus (Northington *et al.*, 2001). Furthermore, hypoxia–ischemia brain injury seems to be attenuated in *Bax* gene-deficient mice compared with wild-type

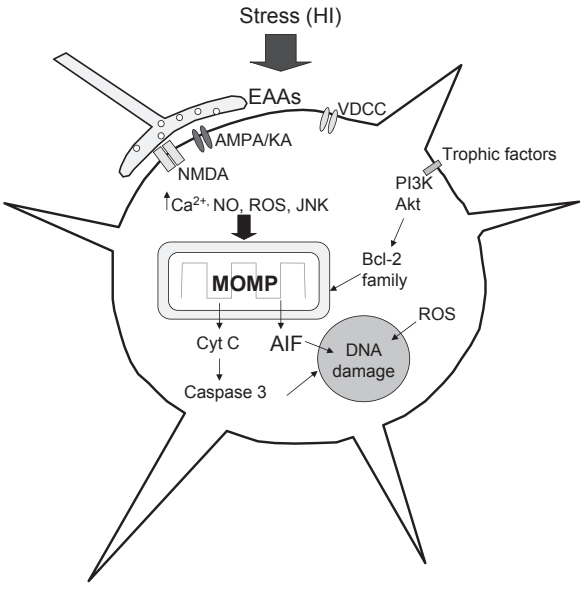

Fig. 17.4 Possible mechanisms for the pathological events contributing to hypoxic–ischemic-induced neuronal death. Hypoxic–ischemic (HI) stress results in excessive excitatory amino acid (EAA) secretion which stimulates α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) glutamate receptors allowing ion transport and depolarization, and activation of second messenger systems that increase intracellular calcium concentrations. Together with accumulated reactive oxygen species (ROS) and nitric oxide (NO) production, and stress-induced transcription factor activation, this leads to mitochondrial dysfunction, release of proapoptotic factors, caspase activation, and DNA damage. MOMP, mitochondrial outer membrane permeabilization; JNK, C-Jun N-terminal kinase; VDCC, voltage-dependent calcium channel; PI3K, phosphoinositide 3-kinase; Cyt C, cytochrome C.

controls (Gibson *et al.*, 2001). Using a site-specific antibody for phosphorylation of Bcl-2 at serine-24 (PS24-Bcl-2), the number of cells positive for PS24-Bcl-2 increased during 3–24 hours of reperfusion in all investigated brain areas after neonatal hypoxia–ischemia (Hallin *et al.*, 2006). Phosphorylation of Bcl-2 coincided with Cyt C translocation and colocalized with, but preceded, caspase 3 activation. In summary, Bcl-2 is phosphorylated (and probably inactivated) and translocated to the nucleus, concomitant with increased mitochondrial Bax immunoreactivity, Cyt C release, and activation of caspase 3.

C-Jun N-terminal kinase 3 (JNK3) in neonatal hypoxia–ischemia

JNK3 is a member of the stress-activated group of mitogen-activated protein kinases (MAPK). The JNK isoform 3 (JNK3) is specifically expressed in the CNS, and stress-induced JNK3 is believed to contribute to brain injury, hence JNK3 deficiency renders adult mice resistant to glutamate excitotoxicity (Yang *et al.*, 1997) and attenuates hypoxic–ischemic damage in juvenile mice (Kuan *et al.*, 2003). We recently found that JNK3 is activated after hypoxia–ischemia and neonatal JNK3-deficient mice are less vulnerable to this (Pirianov *et al.*, 2007). Downstream of JNK3, hypoxia–ischemia resulted in an increased phosphorylation of the transcription factors c-Jun and ATF-2, an effect that was attenuated in JNK3-knockout mice. JNK3 deletion also decreased caspase 3 cleavage and Bim/PUMA expression, coupled with an upregulation of AKT/FOXO3a levels (Pirianov *et al.*, 2007), suggesting that the primary mode of JNK3 action is to promote apoptosis and that JNK3 is acting upstream of mitochondria (Fig. 17.4).

Calpains

Calpains are cysteine proteases involved in signal transduction cascades and differ from caspases as activity is calcium dependent and the proteolysis does not require an aspartic acid residue (Croall & DeMartino, 1991). A high concentration of calcium can contribute to uncontrolled activation of calpains causing degradation of cellular proteins. Calpains have been implicated in both apoptosis and necrosis (Nath *et al.*, 1996), axonal degeneration, and cytoskeletal disruption (Nath *et al.*, 1996; Leist & Nicotera, 1998).

Calpain activity is high in the developing brain especially in the white matter (Croall & DeMartino 1991). Following hypoxia–ischemia, calpains are activated before or together with caspase 3 (Northington *et al.*, 2007; Romanko *et al.*, 2007). Furthermore, synergistic activation of caspase 3 and m-calpains suggests a link between different death signaling following hypoxic–ischemic brain injury (Blomgren *et al.*, 2001). In immature rats, hypoxia–ischemia activates and relocates calpain to the membrane fraction (Ostwald *et al.*, 1993) and

inactivates the endogenous calpain inhibitor calpastatin in vulnerable brain regions (Blomgren *et al.*, 1997). Calpain cleavage products accumulate during delayed cerebral injury, especially in white matter (Blomgren *et al.*, 1995a, b).

Reactive oxygen species

ROS are molecules that contain one or more unpaired electrons (Halliwell, 1992) which makes the free radicals highly reactive and able to disrupt the molecular structure of lipids and proteins with devastating consequences for cellular function (Halliwell, 1992). Depending on the cellular energy balance, ROS can induce both necrosis and apoptosis by mechanisms that involve mitochondrial alterations following hypoxic–ischemic brain damage.

There are several pathways whereby ROS are produced in the brain (Halliwell, 1992). The superoxide radical ($O_2^{\cdot -}$) is produced by: electron leakage from the electron transport chain in mitochondria; oxidation of hypoxanthine to xanthine and urate by xanthine oxidase (mainly in endothelial cells); degradation of free fatty acids by phospholipase A2 into arachidonic acid and subsequent oxidation of arachidonic acid by cyclooxygenase and lipooxygenase; and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) oxidase activity in macrophages, neutrophils, and microglia.

The $O_2^{\cdot -}$ radical has a relatively low reactivity and does not easily cross cell membranes. However $O_2^{\cdot -}$ can react with Fe^{2+} ions and form hydroxyl radicals (·OH) which reacts with almost every molecule at diffusion-limited speed in the presence of transition metals such as Fe^{2+} ions and exerts toxic effects on DNA, activating PARP and depleting cellular NAD^+ and ATP. The ·OH radical initiates lipid peroxidation in a self-perpetuating reaction that disrupts membrane function. Thiol groups on enzymes and structural proteins are oxidized with loss of enzyme function and cytoskeletal disruption (Halliwell, 1992; Palmer, 1995; Perlman, 2006).

There are several defence systems in the brain to reduce the formation of OFRs and several pathways for their inactivation. The $O_2^{\cdot -}$ adduct is dismutated by superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2), which is converted to water and oxygen by either of the two enzymes catalase or glutathione peroxidase. Compounds such as vitamin E (α-tocopherol) act as lipid-soluble scavengers which inhibit lipid peroxidation. Chelation of transition metals such as iron is another endogenous protective mechanism against excessive formation of ROS (Palmer, 1995). Intracellular concentrations of glutathione may be particularly important, and immature oligodendrocytes are especially prone to ROS-induced death because of limited glutathione stores (Wang *et al.*, 2004).

Importantly, immature and adult brain probably have major differences in the handling of free radicals. Scavenging systems may be less developed in immature animals (Saugstad, 1996). Recent studies have demonstrated that many factors including generation of ROS influence the progression of brain injury after hypoxia–ischemia. There is evidence for increased hypoxanthine levels, free radical formation and lipid peroxidation during reperfusion after hypoxia–ischemia in neonatal mice, newborn piglets, immature rats, and fetal sheep (Armstead *et al.*, 1988; Kjellmer *et al.*, 1989; Hasegawa *et al.*, 1991, 1993; Bågenholm *et al.*, 1998).

The neuroprotective strategy of use of free radical inhibitors and scavengers has been evaluated experimentally, but has not been reproduced in the human neonate (Ditelberg *et al.*, 1996; Perlman, 2006). Treatment with the 21-aminosteroid tirilazad mesylate, a lipid peroxidation inhibitor, after hypoxia–ischemia in 7-day-old rats reduces brain damage (Bågenholm *et al.*, 1996). Allopurinol and its metabolite oxypurinol, being inhibitors of xanthine oxidase and ROS scavengers in high concentrations, reduce brain damage when administered before or after hypoxia–ischemia (Palmer *et al.*, 1993). Furthermore, the iron chelator desferrioxamine attenuates hypoxic–ischemic brain damage (Palmer *et al.*, 1994). Recently several antioxidants, such as ascorbic acid, pyrrolidine dithiocarbamate, tanshionine, and melatonin, have been shown to be neuroprotective against hypoxic–ischemic brain injury (Tütüncüler *et al.*, 2005; Xia *et al.*, 2005; Miura *et al.*, 2006; Nurmi *et al.*, 2006). Other experimental data support the concept that ROS production has an important impact on the hypoxic–ischemic responses mediated by hypoxia-inducible factor-1 (HIF-1) and cyclooxygenase-2 (Domoki *et al.*, 2001; Bazan *et al.*, 2002). Therefore, the discovery of therapeutically useful HIF-1 inhibitors in combination with novel specific cyclooxygenase-2 inhibitors holds promise for a new neuroprotective strategy (Martínez-Sanchez & Giuliani, 2007).

Most pharmacological agents penetrate poorly across the blood–brain barrier and it has been

suggested that ROS production is initiated in endothelial and immuno-inflammatory cells from within the vascular compartment (Perlman, 2006) which is supported by the fact that pretreatment with superoxide dismutase (SOD) chelated to polyethyleneglycol (PEG-SOD) affords acute beneficial effects in asphyxiated lambs (Rosenberg *et al.*, 1989) and newborn piglets (Armstead *et al.*, 1992) in spite of no penetrance across the vascular wall (Matsumiya *et al.*, 1991). In adult ischemia neutrophils are a major source of free radical production following hypoxia–ischemia and the major site of action for some neuroprotective free radical scavengers appears to be at the blood–brain barrier (Hall, 1995).

Nitric oxide

As a second messenger, nitric oxide is involved in distinct biological process such as maintenance of blood pressure, defence against microorganisms, and cancer and neurotransmission. On the other hand, nitric oxide has been reported to be involved in brain injury as the inhibition of nitric oxide synthesis attenuated NMDA neurotoxicity (Dawson *et al.*, 1993). Production of nitric oxide, first identified as the endothelium-derived relaxing factor, occurs through conversion of arginine to citrulline by three different nitric oxide synthases: neuronal NOS (nNOS), endothelial NOS (eNOS), and macrophage or inducible NOS (iNOS) (Jaffrey & Snyder, 1995). Both eNOS and nNOS are expressed constitutively but all types of NOS can be induced in response to a variety of stimuli. Both eNOS and nNOS are dependent upon Ca^{2+} binding for activation and nNOS is activated by NMDA receptor stimulation (Eliasson *et al.*, 1997). The activity of iNOS is mainly expressed in inflammatory cells and produces large amounts of nitric oxide and its activity is Ca^{2+} independent (Iadecola & Ross, 1997). Nitric oxide binds to iron and thiol groups including metabolic enzymes and can induce apoptotic and necrotic cell death, with different redox states of nitric oxide (NO^+, $NO^{\cdot}$, and NO^-) having different effects; NO^+ and $NO^{\cdot}$ probably inducing predominantly apoptotic death whereas NO^- precipitates necrosis (Khan *et al.*, 1997). NO. and $O_2^{\cdot -}$ react very quickly to form peroxynitrite ($ONOO^-$), which is freely diffusible, oxidizes thiol groups, and induces protein nitrosylation and mitochondrial impairment, thus contributing to brain damage (Crow & Beckman 1995; Rodrigo *et al.*, 2005).

Investigations into the role of nitric oxide in ischemic brain injury have yielded conflicting results, and the effects of different subtypes of nitric oxide synthases have to be considered separately (Huang *et al.*, 1994; Iadecola & Ross, 1997). In many studies eNOS confers protection through a beneficial vasodilator effect improving perfusion, while nNOS and iNOS enhance injury in response to focal ischemia (Huang *et al.*, 1994). Statins, the most widely used lipid-lowering drugs, have been demonstrated to play a protective role in hypoxic–ischemic stroke through eNOS stimulation in adult brain (Cimino *et al.*, 2005). In addition, hypothermic preconditioning (reduction of temperature to 34 °C) has been shown to reduce damaging iNOS levels and to protect Purkinje cell death (Yuan *et al.*, 2006).

Recent data also suggest that immature brain behaves differently from adult tissue. As in the adult, nitric oxide is produced in increasing amounts during reperfusion (Tan *et al.*, 1996; Thoresen *et al.*, 1997), and some data support a role for nitric oxide and nitric oxide synthases in hypoxic–ischemic injury to the developing brain: selective lesion of cells with NOS activity prior to an hypoxic–ischemic insult decreased brain injury (Ferriero *et al.*, 1995); neonatal mice lacking the gene for nNOS develop smaller brain injury than wild-type mice following hypoxia–ischemia (Ferriero *et al.*, 1996); and non-specific NOS inhibitors provide neuroprotection if administered before the insult (Trifiletti, 1992; Hamada *et al.*, 1994; Ashwal *et al.*, 1995) or in the late reperfusion phase (Palmer *et al.*, 1994) in some studies. However, tissue concentrations of iNOS are very low in immature rat brain and do not appear to be induced within 36 hours of hypoxia–ischemia (Blumberg *et al.*, 1999), and both broad-spectrum and specific NOS inhibition after hypoxia–ischemia are less effective at reducing injury in immature sheep, rats, and mice than in most studies of adult brain (Ferriero *et al.*, 1995; Blumberg *et al.*, 1999; Marks *et al.*, 1999). Indeed NOS inhibition was unable to prevent the secondary energy failure developing after hypoxia–ischemia in immature mice (Blumberg *et al.*, 1999). Equally, intracerebral injection of the nitric oxide donor nitroprusside at doses which inflict damage in the adult brain is not toxic to the neonatal brain (Maragos & Silverstein, 1994) suggesting that the immature brain may be more resistant to nitric oxide toxicity. In addition, very recently it was reported that nitric oxide released from nipradilol, an nitric oxide donor, exerts a neuroprotective effect on hypoxic–ischemic neonatal

neurons (Kakizawa *et al*., 2007). Other drug avenues of potential neuroprotection that have been studied against the toxic effect of nitric oxide in hypoxic–ischemic brain injury include erythropoietin and selective NOS inhibitors and free radical scavengers (Kumral *et al*., 2004; Noor *et al*., 2005). Further work is needed to understand the role of nitric oxide in hypoxic–ischemic encephalopathy.

Inflammatory mechanisms in hypoxic–ischemic injury

Hypoxia–ischemia in the neonatal brain triggers an inflammatory reaction that persists from days to weeks after the initial insult and is thought to play a major role in the progression of injury. The initial pro-inflammatory phase involves the recruitment and activation of a diverse range of immune cells which interact with other cells within the brain parenchyma. However, as the inflammatory process progresses, the proinflammatory phase may be switched to an anti-inflammatory phase to promote tissue repair and recovery.

Microglia, the resident immune cells of the CNS, are intricately involved with the initiation and propagation of the inflammatory response within the brain. Microglia are potent producers of cytokines and other factors that lead to initiation of adaptive immune responses. These cytokines maximize the recruitment of cellular elements to the site of inflammation, however they can also contribute to white matter injury (Bell & Hallenbeck, 2004). Studies suggest that T and B cells, natural killer cells, mast cells, dendritic cells, and polymorphonuclear leukocytes participate in the response to hypoxia–ischemia (Bona *et al*., 1999; Hedtjärn *et al*., 2004). Increase of inflammatory proteins is accompanied by accumulation of neutrophils in the damaged area and activation of microglia/macrophages, lymphocytes, and astrocytes, which may persist for days after the hypoxic–ischemic insult, suggesting a chronic state of inflammation (Bona *et al*., 1999).

Pathological processes in the brain often involve the endothelium and its interactions with circulating blood elements. Neutrophils, monocytes, and platelets are activated by the endothelium and immuno-inflammatory cells activate the endothelium to produce humoral factors and to express adhesion molecules (Akopov *et al*., 1996). These processes have been implicated in adult neurological diseases such as multiple sclerosis, infection, stroke, and trauma (Kochanek & Hallenbeck, 1992) and are likely to play a role in the pathogenesis of injury in the immature brain (Palmer, 1995). Injection of endotoxin into the 7-day-old mouse brain induces a considerable recruitment of polymorphs and a rapid mononuclear response (Lawson & Perry, 1995; Perry *et al*., 1995). Microglial activation occurs early following hypoxia–ischemia or excitotoxicity in the immature rat CNS, and is more rapid in white than in gray matter (McRae *et al*., 1995; Acarin *et al*., 1996; Ivacko *et al*., 1996). In children with early signs of periventricular leukomalacia, activated microglia are prevalent within periventricular lesions and these microglia have strong immunostaining for the pro-inflammatory cytokine tumor necrosis factor (TNF) α (Kadhim *et al*., 2001). The mechanisms whereby immuno-inflammatory cells are activated in response to different insults are not fully understood, but cytokines and chemokines are believed to be involved.

Cytokines and chemokines

Cytokines constitute a large and heterogeneous group of proteins with multiple actions within both the nervous and the immune systems. Among the activities of different cytokines relevant to neurological injury are pro- and antiinflammatory actions, activation of microglia and macrophages, triggering of apoptosis through cell surface receptors, stimulating production of adhesion molecules on endothelium and neutrophil surface, and direct neuroprotective effects (Akopov *et al*., 1996; Thomson, 1998). Chemokines represent a subgroup of cytokines being produced by a variety of immune and nonimmune cells showing chemotactic activity for specific types of leukocytes. The α-chemokines (IL-8, MIP-2, and GRO) mainly attract polymorphonulcear leukocytes whereas -chemokines (MCP-1, RANTES, MIP-1α/) attract and activate macrophages, microglia, and other immune cells.

Cytokines and chemokines are expressed in the immature brain in response to a variety of insults. Interleukin (IL)1 and MCP-1 are expressed after intracerebral injection of NMDA (Hagan *et al*., 1996; Szaflarski *et al*., 1998), and IL1α, IL1 , TNFα, IL6, MIP1α, MIP1 , MIP2, RANTES, GRO, and MCP1 are all induced after hypoxia–ischemia in neonatal rats (Szaflarski *et al*., 1995; Hagberg *et al*., 1996; Ivacko *et al*., 1997; Bona *et al*., 1999; Hedtjärn *et al*., 2004). However, despite intense interest, the association of hypoxia–ischemia and release of cytokines is still not fully understood. Studies using experimental

models have suggested that cytokines such as IL6 play a complex role in hypoxia–ischemia, capable of mediating both damage and protection (Hagberg *et al.*, 1996; Loddick *et al.*, 1998; Dihne & Block, 2001) and that IL6 and TNFα contribute to the brain injury in perinatal asphyxia (Martín-Ancel *et al.*, 1997; Oygür *et al.*, 1998; Shalak *et al.*, 2002). At a clinical level, there is a direct correlation between CSF levels of IL6 and TNFα and the prognosis in acute brain ischemia in adults (Vila *et al.*, 2000). High CSF levels of IL6 have been observed in term newborn infants with severe hypoxic–ischemic encephalopathy (Martín-Ancel *et al.*, 1997) and higher CSF concentrations of IL1 , IL6, and TNFα are correlated with the clinical severity of asphyxia (Aly *et al.*, 2006). In longer-term studies, increased cerebrospinal fluid (CSF) levels of TNFα and IL1 after perinatal hypoxic insult were associated with neurologic alterations at 12 months of age (Oygür *et al.*, 1998). In addition, elevated serum levels of IL1 , IL6, and TNFα were found in newborn infants with changes characteristic of encephalopathy on MRI and who developed cerebral palsy (Foster-Barber *et al.*, 2001). It is thought that the increase in CSF cytokine levels in newborn infants with hypoxic–ischemic encephalopathy result from direct effects of asphyxia on the CNS and are not due to generalized disruption of the blood–brain barrier (Silveira & Procianoy, 2003).

The involvement of inflammatory mediators in the progression of ischemic injury has been shown by a number of studies in which neuroprotection was achieved by intervening with their functions. However, the exact pathogenetic mechanism that leads to cytokine release is not completely known. It has been suggested that necrotic cells and damaged tissues during ischemia release molecules (i.e., fibrinogen, DNA) that could activate the inflammatory pathway through binding of toll-like receptors leading simultaneously to the release of proinflammatory cytokines such as TNFα, IL1 , and IL6, and antiinflammatory cytokines such as IL4, IL10, and IL13. The activation of both pro- and antiinflammatory molecules by the same initial signal may modulate the extension and severity of inflammatory damage (Paterson *et al.*, 2003; Yang *et al.*, 2004).

Toll-like receptors (TLRs) are a family of signal transduction molecules that play a critical role in the induction of innate and adaptive immunity. Studies have shown that activation of one member of this family, TLR4, plays a role in ischemia/reperfusion injury (Hua *et al.*, 2007). Activation of TLR4 results in the nuclear translocation of the transcription factor nuclear factor (NF)- B and the transcription of genes involved in the inflammatory process leading to release of proinflammatory cytokines. The expression of TLR4 is enhanced in neonatal rat brain following hypoxia–ischemia (Maslinska *et al.*, 2004). TLR4-deficient mice have a decreased expression of proinflammatory cytokines, less neuronal cell death, and reduced infarct size following ischemia (Cao *et al.*, 2006; Caso *et al.*, 2007; Hua *et al.*, 2007). However, the mechanism by which TLR4 signaling induces neuronal death remains unknown.

The inflammatory pathway induced after hypoxia–ischemia may also be activated following the release of cathepsin B into the cytoplasm, which may activate the caspase cascade by the processing of caspases which are involved in the maturation of the functional forms of cytokines IL1 and IL18 (Benchoua *et al.*, 2004). Activation of these cytokines could play a role in promoting the inflammatory response following hypoxia–ischemia and also modulate post-injury apoptosis (Dietrich *et al.*, 2004).

Cytokines also stimulate microglia and astrocytes to produce other cytokines, chemokines, nitric oxide, EAAs, and ROS, which are particularly detrimental to the immature brain due to the enhanced vulnerability of maturing cells. Therefore, cytokines in preterm infants in the context of inflammation could act not only as mediators of damage but also as a dysregulated network of neurotrophic factors that alter the fragile mechanisms of CNS development. In support of this, cytokines inhibit the differentiation and proliferation of oligodendrocyte precursor cells, which affects active myelination and may lead to white matter injury (Kinney & Back, 1998; Pang *et al.*, 2003). Taken together, evidence suggests several potential mechanisms through which cytokines may lead to or exacerbate neonatal brain injury following hypoxia–ischemia.

The role of cytokines and chemokines in perinatal brain injury is highly complex as the example of TNFα demonstrates. In some situations TNFα is toxic to neural cells: it exerts toxic effects on neurons (Gelbard *et al.*, 1993; Taylor *et al.*, 2005) and oligodendrocytes (Robbins *et al.*, 1987; Selmaj & Raine, 1988), inducing apoptosis in oligodendrocytes (Leist & Nicotera, 1998) by receptor-mediated activation of caspase 8 (Cohen, 1997). Administration of exogenous TNFα markedly exacerbates ischemic injury (Yoon *et al.*, 1997). Both TNFα-binding

protein (which prevents binding to cell surface receptors in this system) and anti-TNFα antibodies reduce ischemic injury (Shohami *et al.*, 1996; Nawashiro *et al.*, 1997; Lavine *et al.*, 1998). These observations offer support for the assumption that TNFα exerts injurious effects. However, in other situations TNFα is protective; protecting cultured neurons from the toxicity of -amyloid and making astrocytes resistant to acidosis and calcium ionophore toxicity, indeed TNFα-knockout mice are more prone to extensive inflammation and demyelination (Liu *et al.*, 1998). This protective action may be mediated through the TNF p55 receptor, and genetic deletion of both TNF receptors (p55 and p75) increased the extent of ischemic and excitotoxic injury (Bruce *et al.*, 1996).

Other cytokines are less well studied. Production of IL1 is essential for brain injury in animal studies since deficiency of IL1 -converting enzyme or treatment with IL1 receptor antagonist reduced brain injury in immature rats subjected to hypoxia–ischemia (Martin *et al.*, 1994; Hagberg *et al.*, 1996). IL18, which is produced by astrocytes and microglia, plays a role in hypoxic–ischemic-induced damage after hypoxia–ischemia since mice deficient in IL18 showed reduced injury (Hedtjärn *et al.*, 2002). In the case of IL6, the exact role of this cytokine in hypoxic–ischemic encephalopathy remains unclear. An antiinflammatory role has been suggested since IL6 opposes the effects of TNFα and IL1 by both inhibiting their synthesis and stimulating the generation of their naturally occurring antagonists. As such, IL6 may have both neurotrophic and neuroprotective effects and its production may be involved in the repair process after ischemic brain injury (Loddick *et al.*, 1998; Chiesa *et al.*, 2003).

Conclusion

The mechanisms of cerebral injury in hypoxic–ischemic encephalopathy are multiple and complex. The realization that brain injury develops over a considerable period of time, and that active cell processes such as apoptosis and inflammation are important, has led to important advances, not least the belief that treatment of hypoxia–ischemia might be successfully initiated after resuscitation from the insult. The apparent success therapy of moderate hypothermia as a neural rescue therapy seems to be valuable proof of concept that treatment is possible, but clinical trials of more targeted neural rescue therapies are only just beginning and potential treatments have yet to prove themselves. Further advances in basic understanding of the mechanisms of injury, possibly in the elucidation of the role of inflammatory responses in hypoxic–ischemic injury, may significantly improve our chances of developing successful treatments for hypoxic–ischemic encephalopathy.

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Chapter 18

Clinical assessment and therapeutic interventions for hypoxic–ischemic encephalopathy in the full-term infant

Andrew Whitelaw and Marianne Thoresen

Definition and diagnostic criteria

The term hypoxic–ischemic encephalopathy (HIE) implies a clinically apparent acute disturbance in brain function resulting from a period of critical deprivation of cerebral oxygen delivery and/or blood supply. For the diagnosis of HIE to be upheld there needs to have been obstetric evidence of risk of hypoxia/ischemia to the fetus (e.g., reduced fetal movements, prolapse of the umbilical cord, late decelerations of the fetal heart, fresh meconium, placental abruption, etc.) after which the infant is born in poor condition, with delayed onset of respiration, and is then observed to have cerebral dysfunction (e.g., hypotonia, inability to suck, abnormal posture, clonic movements).

The finding of metabolic acidosis or raised lactate levels in cord blood or blood taken within 30 minutes of birth provides important supporting evidence that there has been acute hypoxia–ischemia at or shortly before delivery. Further support for a global hypoxic–ischemic episode is provided by deranged liver function (raised transaminases), a period of renal impairment with oliguria and raised serum creatinine, cardiac dysfunction, and disseminated intravascular coagulation. The diagnosis of HIE also requires that steps have been taken to rule out other causes of cerebral dysfunction such as infection, preexisting anatomical abnormalities of the brain, or an inherited metabolic disease.

As it is often difficult to be sure if hypoxia–ischemia is the cause of encephalopathy, some authors prefer the term neonatal encephalopathy, which makes no assumptions or exclusions as to etiology. Thus, meningitis, urea cycle defects, nonketotic hyperglycinemia, and cerebral migration disorders would all be included in neonatal encephalopathy. We have investigated cases of moderate and severe encephalopathy at term over an eight-year period and found a clear majority to have evidence of hypoxic–ischemic etiology (Draycott *et al.*, 2006).

Clinical assessment of HIE

Clinical features

During resuscitation after perinatal hypoxia, the infant is typically hypotonic, and unresponsive. The initial response is in the heart rate and blood pressure followed by improvement in skin color. Following resuscitation, there may be a period of mild to moderate neurological signs, but severe neurological dysfunction may also be apparent from the start. There is usually a sequence of clinical features and it is possible to grade the severity over a period of 48 hours. Clinical assessment of the neurological status of sick newborn infants is based on:

- observation of spontaneous movements:
  - respiration – regular/irregular/apnea?
  - eyes open/closed
  - eye movements
  - posture – is the neck held in extension? are the legs flexed or straight?
  - character and quality of limb movements – tremors? clonic movements?
  - facial expression – spontaneous crying? does the baby seem irritable?
- response to light and sound;
- sucking and swallowing;
- passive tone in the limbs and trunk;

The Newborn Brain: Neuroscience and Clinical Applications, 2nd edn., eds. Hugo Lagercrantz, M. A. Hanson, Laura R. Ment, and Donald M. Peebles. Published by Cambridge University Press.

- active tone in the limbs and trunk including Moro reflex, grasp and deep tendon reflexes at the knee and ankle.

One physical sign which is often observed in moderate HIE is neck extensor hypertonia (Amiel-Tison, 2008). The examiner holds the infant's shoulders and pulls the infant from the lying to the sitting position, noting the position of the head in relation to the trunk. Normally this elicits active contraction of the neck flexors with the head flexed forward on to the chest (Fig. 18.la). The examiner then moves the trunk gently backwards. Normally there is contraction of the neck extensors tending to lift the head before the trunk gets to the vertical position. In the normal term infant, flexion of the neck and extension are equally strong. Figure 18.1b shows the effect of global hypotonia or weakness. There is no active contraction of the neck muscles either way. In Figure 18.1c the head is held extended even when the trunk is flexed forward and the chin is poking out. This neck extensor hypertonia is illustrated in Fig. 18.2a.

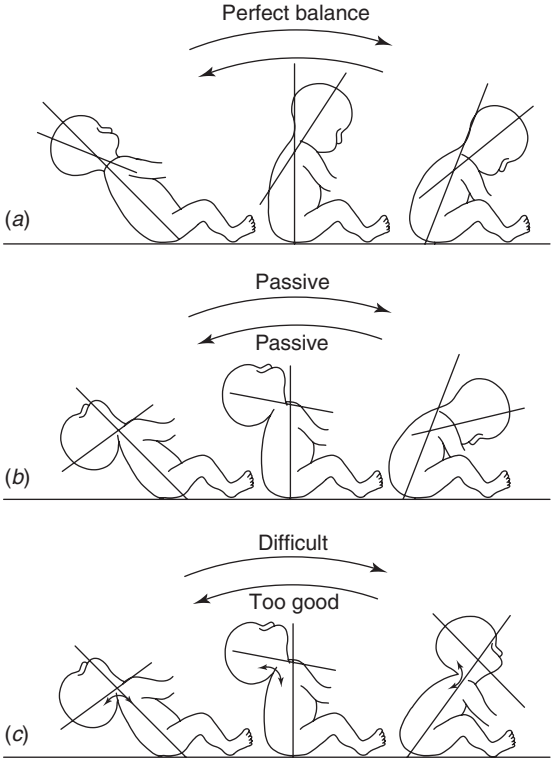

Fig. 18.l (*a*) Holding the shoulders and moving the trunk forwards and then backwards. Normally flexion of the neck is equally strong as is extension of the neck. (*b*) If there is global weakness or hypotonia, the head hangs back when the trunk is held back and the head hangs forward when the trunk is held forward. (*c*) When there is neck extensor hypertonia, the head is still held back even when the trunk is held forward. (From Amiel-Tison, C. & Gosselin, J., 2008 with permission from Elsveier. © 2008 Elsevier.)

Neck extensor hypertonia can also be observed, to some extent, when one holds the infant in ventral suspension. Besides being common in moderate HIE, this finding is also frequent in full-term infants with blood in the subarachnoid space. Figure 18.2b shows an infant with more signs of moderate HIE. The legs are extended and adducted (scissor position). The hands are held fisted with the thumbs adducted. The head is held slightly extended. The eyes are open but there is no fixation or following. It is very important to listen to the nurse's description and impression of the baby's alertness, irritability/consolability, tone, and movement quality.

Grading encephalopathy

It is usually possible by a combination of observation and examination to see a pattern that enables one to classify the affected baby as mildly, moderately or severely encephalopathic. Sarnat and Sarnat (1976) first classified postasphyxial encephalopathy into three grades at 24 hours of age (Table 18.1). This approach has been used with minor modifications by others such as Amiel-Tison and Ellison (1986), Finer *et al.* (1983), Fenichel (1983), Levene *et al.* (1986), and Thompson *et al.* (1997). The clinical picture may be dominated early by tachypnea, resulting from intracerebral lactate, and this may be sufficient to cause hypocapnia. This may be combined with periodic breathing and apnea. The essential features of mild encephalopathy are minor changes in tone, behavior, and responsiveness. Moderate encephalopathy is characterized by marked disturbances in tone, posture, and ability to feed. Convulsions may or may not occur. Severe encephalopathy is characterized by loss of most or all reflexes and responsiveness. If seizures occur they are difficult to treat. From the clinician's point of view it is important to determine whether the infant is deteriorating from moderate encephalopathy to severe encephalopathy. Loss of reflex activity including respiratory failure, pooling of saliva and the loss of the doll eye movements are important signs of severe encephalopathy.

Grade of encephalopathy and prognosis

The largest study examining the grade of HIE and neurological follow-up is by Robertson and Finer (1985). They examined survivors at 3.5 years and documented deaths among 226 infants with HIE. Of

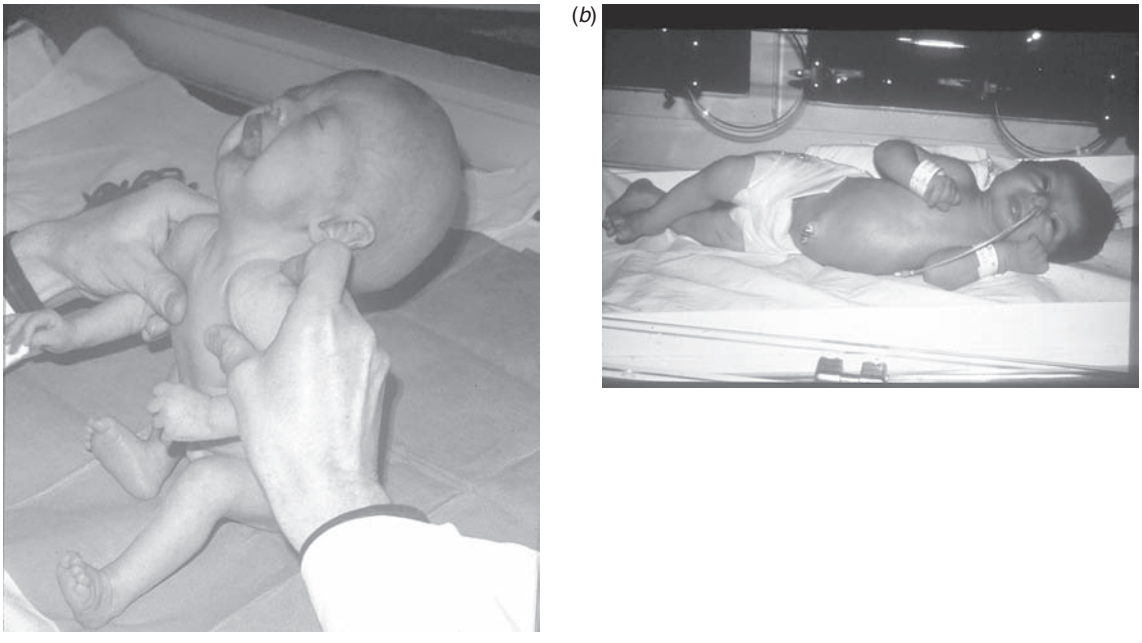

Fig.18.2 (*a*) An infant with neck extensor hypertonia. (*b*) An infant with moderate hypoxemic–ischemic encephalopathy (HIE). The legs are held extended and adducted (scissor position). The hands are held fisted and the head is slightly extended. The eyes are open but without fixation or following.

79 infants with mild HIE, no child died or survived with disability. Of 119 with moderate HIE, 5% died and 21% of survivors were disabled. Of 28 with severe HIE, 75% died and 100% of the survivors were disabled mostly with spastic cerebral palsy and cognitive disturbances.

The duration of encephalopathy also helps with prognosis. In Sarnat and Sarnat's 1976 study, infants who did not enter stage 3 and had signs of stage 2 for less than five days were normal at follow-up but persistence of stage 2 for more than seven days was associated with neurological sequelae or death. Other studies have found that infants with a normal neurological examination by about one week were grossly normal at follow-up (Scott, 1976; Robertson & Finer, 1985).

Clinical seizures

Clinical seizures occur during the 6–12 hours after birth in over half of the infants who will eventually develop seizures (Volpe, 2001). Recognition of seizure activity in newborns can be difficult but focal or multifocal clonic movements which are sustained and rhythmic, not stopped by restraint, have a fast component and a slow component, and are accompanied by deviation of the eyes and disturbance of breathing are very likely to correlate with electrical seizure activity. It has become apparent that many episodes of seizure activity on electroencephalography (EEG) are not accompanied by clinical seizure activity (Eyre *et al.*, 1983). Episodes of bilateral extensor tonus look very dramatic and are certainly an abnormal neurological sign but they correlate only rarely with EEG epileptic activity (Volpe, 2001).

EEG and prognosis

The electroencephalogram of an awake term infant is characterized by continuous activity with variable amplitude (usually in the range 10–25 μV) and variable frequencies (Fig. 18.3a). During quiet sleep there are brief periods (<10 seconds) of lower (down to 7.5 μV but not very low) amplitude alternating with periods of higher amplitude. A cerebral insult to a term infant results in the EEG becoming discontinuous with periods of normal amplitude alternating with periods of much lower voltage (<5 μV). Eventually the EEG may be nearly all very low voltage with periodic short bursts of higher voltage activity. A more serious cerebral insult results in all voltages being reduced even when the EEG is active. These gradations in EEG are well presented to bedside clinicians by the amplitude-integrated EEG (aEEG) or

Table 18.1 A clinical grading system for postasphyxial encephalopathy

	Postasphyxial encephalopathy		
	Mild	**Moderate**	**Severe**
Level of consciousness	Hyperalert	Lethargic	Stuporous
Tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch reflexes	Overactive	Overactive	Decreased or absent
Segmental myoclonus	Present	Present	Absent
Complex reflexes			
Suck	Weak	Weak or absent	Absent
Moro	Strong: low threshold	Weak: incomplete; high threshold	Absent
Oculovestibular	Normal	Overactive	Weak or absent
Tonic neck	Slight	Strong	Absent
Autonomic function	Generalized sympathetic	Generalized parasympathetic	Both systems depressed
Pupils	Mydriasis	Miosis	Variable; often unequal; often poor light reflex
Heart rate	Tachycardia	Bradycardia	Variable
Salivary secretions	Sparse	Profuse	Variable
Gastrointestinal motility	Normal or decreased	Increased; diarrhea	Variable
Seizures	None	Common; focal or multifocal	Uncommon (excluding decerebration)
EEG	Normal	Early: low-voltage continuous; spike and wave	Burst suppression or iso-potential

Source: Sarnat & Sarnat (1976).

cerebral function monitor (CFM), which displays one or two channels of EEG compressed to 6 cm/h instead of conventional EEG's 3 cm/s. In addition aEEG rectifies the signal so that everything is displayed above zero, instead of crossing zero-like conventional EEG. Modern aEEG equipment displays "raw" EEG as well as aEEG.

The aEEG signal gives very useful information in real time on the severity of brain dysfunction and the prognosis but it can sometimes be difficult to be certain about epileptic activity on the CFM without seeing the "raw" EEG. Nevertheless, the ease of application, relatively small size, and robustness of the CFM makes it acceptable to junior medical and nursing staff. The finding of continuously low-amplitude EEG or burst suppression is of great importance prognostically and had a positive predictive value for death or disability of 84.2% and a negative predictive value of 91.7% at six hours (Eken *et al.*, 1995). A very low amplitude or isoelectric recording at three or six hours resulted in death or disability in all cases. Figure 18.3b shows aEEG recording from an infant with severe HIE at about six hours. The upper margin is reduced below 10 μV and the lower margin of the trace is very low, about 1 μV with intermittent short "bursts" of higher amplitude. This is burst suppression. The aEEG did not normalize during the first 48 hours and this child went on to develop severe motor problems. Burst suppression before six hours may, in about 25% of cases, revert to continuous activity by 24 hours with the possibility of normal outcome (Helström-Westas *et al.*, 1995; Toet *et al.*, 1999). In some cases electrical seizures are superimposed on a burst suppression pattern (Fig. 18.3c). In moderate HIE, the EEG recording may show a discontinuous pattern with normal voltage during active phases (Fig. 18.3d) or may pass through this phase on the way to recovering to a normal continuous normal voltage.

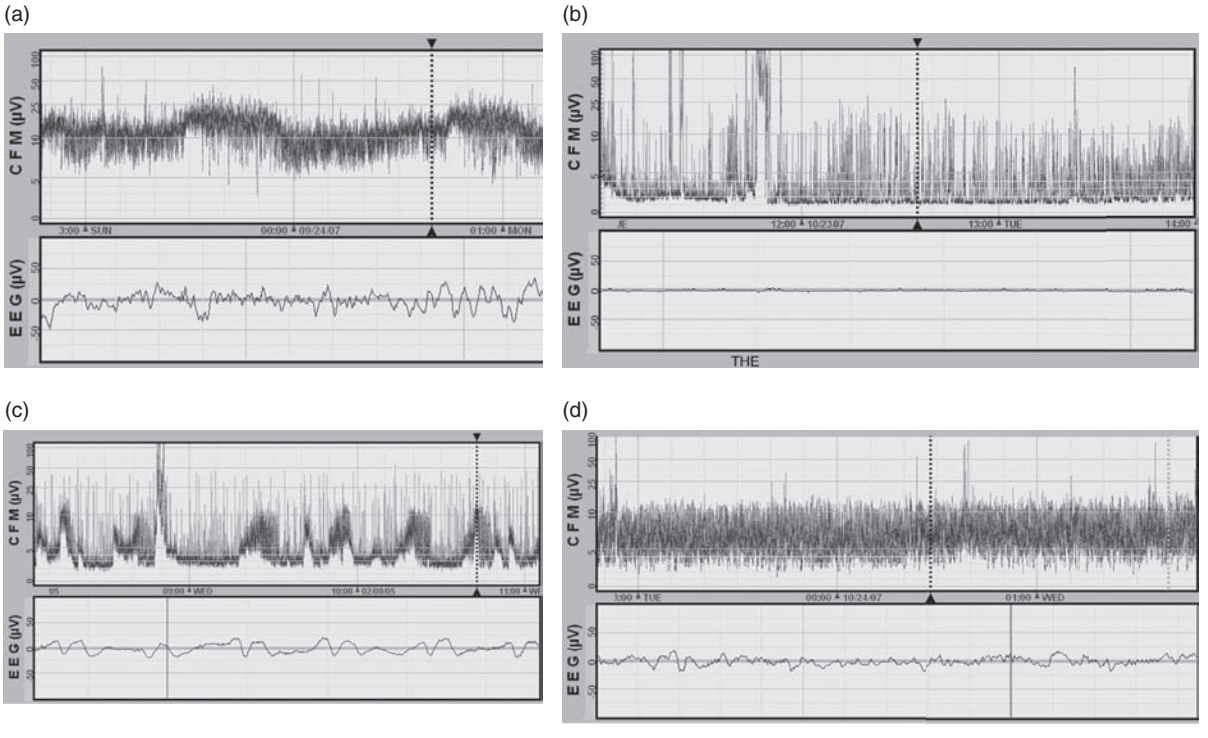

Fig. 18.3 (*a*) Continuous normal voltage amplitude-integrated EEG (aEEG) with the upper margin above 10 μV and the lower margin above 5 μV (three hours in the top panel) and, in the lower panel, seven seconds of raw EEG sampled where the aEEG is marked. (*b*) Severe hypoxemic–ischemic encephalopathy (HIE). Burst suppression aEEG with the upper margin 1–2 μV and intermittent bursts above 10 μV in the top panel and essentially flat EEG in the lower panel sampled where the aEEG is marked. (*c*) Severe HIE. Burst suppression with seizures. The aEEG shows sudden elevations of the lower margin and the "raw" EEG shows rhythmic discharges. (*d*) Moderate HIE. Discontinuous aEEG with the upper margin above 10 μV and the lower margin below 5 μV. In the lower panel, the "raw" EEG is below.

In the piglet model of global hypoxia, the severity of the hypoxic insult and the ultimate brain injury correlated with the duration of low-amplitude EEG during the insult, the time before spontaneous respiration and the time before return of EEG activity (Thoresen *et al.*, 1996a). For more details see Chapters 14 and 15.

Biochemical markers

Severe hypoxia produces characteristic metabolic derangements that help in the clinical assessment if the analyses can be carried out rapidly. The magnitude of the metabolic acidosis in cord blood or in the first hour or two is confirmation of an acute intrapartum hypoxic insult. Indeed, marked neurological abnormality with normal acid–base status in cord blood is good evidence that the insult was probably a considerable time before delivery. There is an association between umbilical artery acidosis (pH<7.0) and HIE (Goodwin *et al.*, 1992) and neurological deficit at one year (Low *et al.*, 1988) although it is important to point out that the majority of fetuses with umbilical artery pH <7.0 were normal later. A number of metabolites, brain proteins, and enzymes that can be measured in blood, urine, or cerebrospinal fluid have been shown to correlate with HIE and neurological deficit, but as they are not routinely available in clinical laboratories they will not be considered further. One available biochemical marker is the lactate/creatinine ratio in urine at six hours, which is predictive of HIE (Huang *et al.*, 1999).

Ultrasound and prognosis

Cranial ultrasound is helpful in the assessment of HIE, as it can be carried out at the cot side with no disturbance and no ionizing radiation to the infant. Ultrasound may show increased echogenicity in the basal ganglia and thalamus as seen in Fig. 18.4 but is usually normal in the first six hours. If there is severe cerebral edema, a generalized echogenicity with loss of details of the cerebral structures (bright brain) may

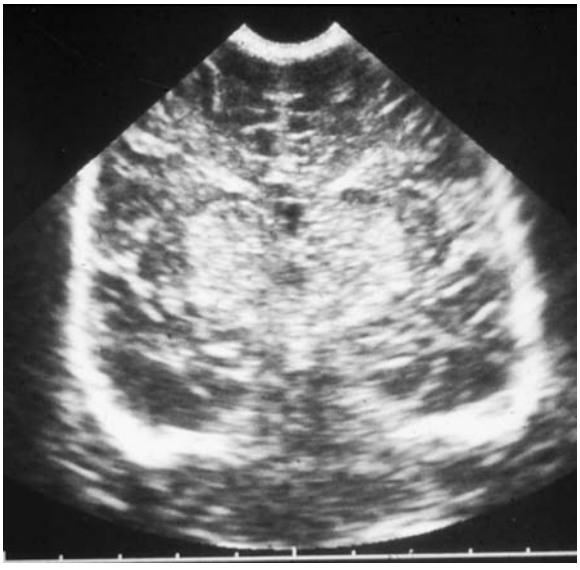

Fig. 18.4 Severe hypoxemic–ischemic encephalopathy (same baby as in Fig. 18.6). Cerebral ultrasound (coronal view) showing increased echogenicity in the basal ganglia and thalamus.

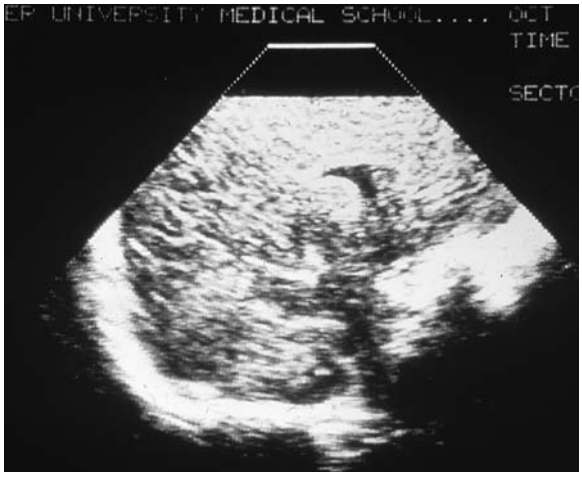

Fig. 18.5 Severe hypoxemic–ischemic encephalopathy. Cerebral ultrasound (sagittal view) showing di use echogenicity throughout the brain substance.

be seen, as in Fig. 18.5. Another important function of an early ultrasound examination is the ruling out of gross congenital abnormalities such as holoprosencephaly or agenesis of the corpus callosum and antenatal abnormalities such as ventricular dilatation. Ultrasound, in our experience, is less helpful than clinical examination and aEEG in assessing the severity and prognosis of HIE.

Cerebral Doppler and prognosis

Cerebral blood flow velocity studies of the middle cerebral artery have shown that a low resistance index ([systolic velocity–diastolic velocity]/systolic velocity) that is below 0.55 after 24 hours is highly predictive of poor neurological outcome (Archer *et al.*, 1986). Rather than demonstrating a late no-reflow phenomenon, infants with severe HIE tend to show increased blood flow (luxury perfusion). Thus a low resistance index after 24 hours is a feature of cerebral vasodilatation with uncoupling of cerebral blood flow to metabolism.

Cranial computed tomography and prognosis

Computed tomography (CT) findings in HIE are discussed in Chapter 13 but CT, in our experience, is not helpful in assessing prognosis early.

Magnetic resonance imaging and prognosis

Magnetic resonance imaging (MRI) is a challenging examination in a critically ill infant but is becoming increasingly valuable, and abnormal signal density in the posterior limb of the internal capsule at about 4–17 days of age in infants with HIE has been shown to be 100% predictive of neurological abnormality at 1 year of age. Negative predictive value was 0.87. Sensitivity was 0.9 and specificity was 1.0 (Rutherford *et al.*, 1998). Abnormal signal in the basal ganglia, thalamus, and brainstem on MRl is also associated with a bad prognosis (Fig. 18.6). Diffusion-weighted imaging can demonstrate ischemic lesions earlier (Bydder *et al.*, 2001). MRl is the best method of revealing or excluding prenatal or congenital abnormalities of the brain including migration disorders. This is extensively described in Chapter 13.

Interventions

Mannitol

Cerebral edema often follows severe cerebral hypoxia–ischemia in adults and is common in severe HIE in infants (Fig. 18.7). Mannitol acts as an osmotic agent drawing water out of the brain and thus reducing edema. This effect has often been used in adult neurosurgical practice to reduce raised intracranial pressure (ICP). Marchal and colleagues (1974) reported an uncontrolled study of 225 asphyxiated full-term

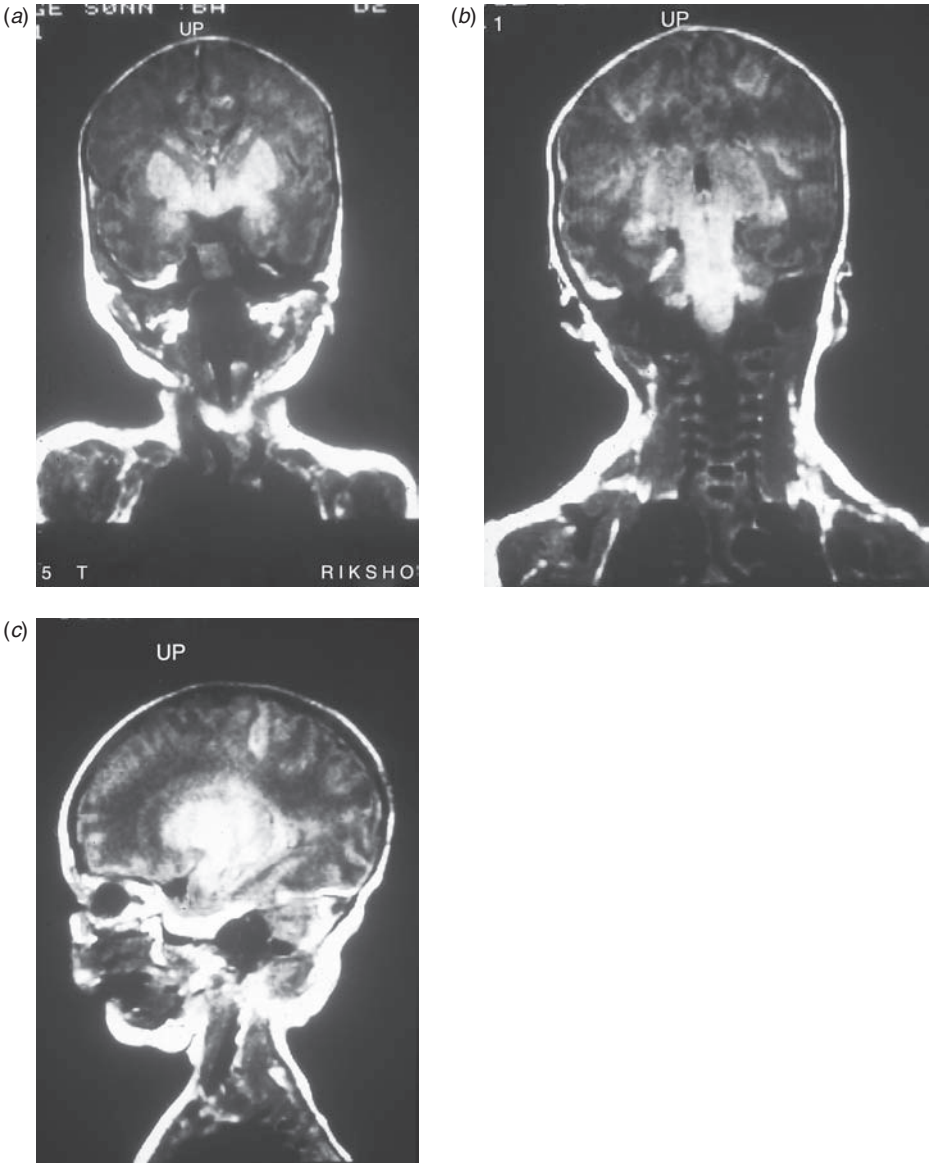

Fig. 18.6 Severe hypoxemic–ischemic encephalopathy (same baby as in Fig. 18.4). Cerebral magnetic resonance T1-weighted image. (*a*) Coronal view: abnormal bright signal in the basal ganglia and thalami. (*b*) Coronal view: abnormal bright signal in the brainstem and basal ganglia. (*c*) Sagittal view: abnormal bright signal in basal ganglia and thalamus.

infants. The infants qualified for the study if they had an Apgar score of 7 or less at five minutes. Others had neurological findings and many of the infants had both; 130 of the infants received 1g/kg intravenous mannitol before two hours and 95 received mannitol after two hours. There was a lower proportion of deaths and of disabled infants at one year in the group who received mannitol before two hours but, as the groups were not randomized, no conclusion about the efficacy of early mannitol is justified.

Levene and Evans (1985) studied the effect of mannitol in asphyxiated infants who developed HIE and had raised ICP (>10 mmHg measured by subarachnoid catheter) which had not resolved despite dexamethasone. Mannitol intravenous infusion 1 g/kg produced a fall in ICP in four infants and an improvement in cerebral perfusion pressure within 60 minutes, but in a larger series all nine infants with sustained ICP >15 mmHg died or survived with disability inspite of mannitol (Levene *et al.*, 1987). There is no

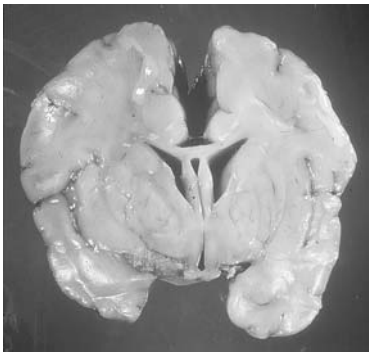

Fig. 18.7 Postmortem brain after severe hypoxemic–ischemic encephalopathy. The hemispheres are swollen, the surfaces being flattened.

randomized trial of mannitol in neonatal HIE but the observations that some infants develop severe HIE without marked rises in ICP and the finding that raised ICP tends to occur after many hours of encephalopathy suggest that raised ICP from cerebral edema is probably a marker that severe brain damage has occurred rather than a mechanism by which damage is caused.

Mujsce and colleagues (1990) studied the efficacy of mannitol on 7-day-old rats subjected to unilateral carotid artery occlusion and 8% oxygen for three hours; 27 rats were given mannitol subcutaneously immediately after the cerebral hypoxia–ischemia and mannitol was repeated every 12 hours for a total of four doses. Control animals received either nothing or normal saline. Mannitol without hypoxia produced no deaths but increased serum osmolality from 287 mosmol/l to 361 mosmol/l. Mannitol significantly reduced brain water content but did not reduce the incidence, distribution, or severity of tissue injury in the cortex, subcortical white matter, hippocampus, or thalamus. Thus, the routine use of mannitol is not justified in neonatal HIE.

Glucocorticoids

Glucocorticoids have been used to reduce edema surrounding brain tumors. Because of this, glucocorticoids, particularly dexamethasone, began to be used in the management of HIE, in order to prevent or reduce cerebral edema. No randomized trials have evaluated the effect of dexamethasone on neonatal HIE but Svenningsen and colleagues (1982) included 2 mg of dexamethasone six hourly in "brain-oriented intensive care treatment in severe perinatal asphyxia." Because the study used historical controls and several interventions were introduced together, it is impossible to conclude that dexamethasone had a positive effect on outcome.

Levene and Evans (1985) measured intracranial and arterial pressure invasively in infants with HIE and gave dexamethasone 4 mg if ICP rose above 10 mmHg. There was a fall in ICP within one hour but this was accompanied by a fall in arterial pressure so that cerebral perfusion pressure was not improved.

The 7-day-old rat cerebral hypoxia–ischemia model with unilateral carotid ligation and several hours of hypoxia has been used to evaluate dexamethasone. Barks and colleagues (1991) found that dexamethasone given 24 hours before the insult was protective but treatment after the insult was not protective. Altman and colleagues (1984) showed in the same model that dexamethasone given immediately before the hypoxic insult did not alter the pattern of neuropathological damage or reduce the fall in high-energy phosphates. Furthermore high-dose dexamethasone (40 mg/kg) was associated with an increase in mortality when compared to low-dose (4 mg/kg) or no-dose dexamethasone. There is also considerable evidence that corticosteroid administration in early life restricts brain growth and development (Howard, 1968) and can increase disability (Shinwell *et al.*, 2000). When all the above evidence is combined with the lack of evidence that glucocorticoids improve outcome in adult patients with hypoxic–ischemic injury or head trauma (Fishman, 1982; Dearden *et al.*, 1986), glucocorticoids cannot be recommended as therapy for neonatal HIE.

Barbiturates

Seizures are frequently present in moderate and severe HIE. Some clinical seizures cause disturbances in respiration and blood pressure which may worsen the cerebral injury. In addition, there is evidence that prolonged seizure activity may produce cerebral injury in its own right although the evidence for this in the newborn brain is inconsistent (Soderfeldt *et al.*, 1990). Seizures experimentally induced in normal brain by chemicals increase cerebral lactate concentrations greatly but posthypoxic seizures in the neonatal piglet are not accompanied by a further increase in cerebral lactate (Thoresen *et al.*, 1998). This may be because lactate is itself used as a fuel by the neonatal brain.

In addition to anticonvulsant activity, barbiturates decrease cerebral metabolic rate and scavenge free radicals. Barbiturates have been proposed as agents

Table 18.2 Barbiturate versus control after severe perinatal asphyxia

Study	Barbiturate	Control	Relative risk (95% CI)
Outcome: Severe neurodevelopmental disability in survivors examined/total			
Goldberg *et al.*, 1986	5/11	6/11	0.83 (0.36 to 1.94)
Ruth *et al.*, 1991	3/16	2/14	1.31 (0.25 to 6.76)
Hall *et al.*, 1998	1/13	6/12	0.15 (0.02 to 1.1)
Outcome: Death or severe neurodevelopmental disability/total			
Goldberg *et al.*, 1986	10/17	9/15	0.98 (0.55 to 1.74)
Ruth *et al.*, 1991	8121	5/17	1.3 (0.52 to 3.24)
Hall *et al.*, 1998	3/20	10/20	0.3 (0.1 to 0.93)

Modified from Evans *et al.* (2007)

that might reduce brain injury in neonatal HIE if given prophylactically without waiting for clinical seizure activity. Three randomized trials have compared prophylactic barbiturate therapy with no routine anticonvulsant therapy in full-term neonates with severe asphyxia. Goldberg and colleagues (1986) gave thiopental 25 mg/kg over 20 hours as the initial intervention to infants with poor Apgar scores and early signs of HIE. Ruth and colleagues (1991) gave 30 mg/kg intravenous phenobarbital then 15 mg/kg four hours later to infants with Apgar score <4 at five minutes or ventilator dependent for more than 30 minutes. Hall and colleagues (1998) gave 40 mg/kg intravenously on the basis of Apgar score at 10 minutes of age. The results of the three trials are shown in Table 18.2.

Close examination of the three trials shows potential sources of bias. The method of allocation concealment is not clear in the studies by Ruth and Goldberg. None of the trials used a placebo and thus all the clinical staff knew which infants received the barbiturate, raising the possibility that infants who had received the active therapy might have been treated differently (consciously or subconsciously) in other ways, e.g., more (or less) careful attention to oxygenation, blood pressure, pCO_2, blood glucose sterile technique, or temperature. Many infants in the control group developed clinical seizures and were then treated with anticonvulsant therapy, usually phenobarbital initially. Thus in Hall's trial the control group received a mean of 27 mg/kg of phenobarbital, although this was started much later than the larger dose of phenobarbital given to the active treatment group. There was significant loss during follow-up, 3% in Goldberg's trial and 23% in Hall's trial, and Ruth did not state the number lost to follow-up. Only one study (Hall *et al.*, 1998) showed a reduction in relative risk of severe developmental disability or death in the barbiturate-treated group. The loss to follow-up and the lack of allocation concealment detract somewhat from the strength of the conclusion. The metaanalysis of all three trials involved only 77 infants and showed no significant effect on death, severe neurodevelopmental disability or death or severe neurodevelopmental disability. High-dose barbiturate therapy can also produce significant reductions in blood pressure in asphyxiated infants (Eyre & Wilkinson, 1986; Goldberg *et al.*, 1986). Thus prophylactic therapy with barbiturates cannot be recommended for infants with severe birth asphyxia or early HIE on present evidence.

Calcium channel blockers

Following hypoxia, reoxygenation is followed by a cascade of cellular reactions which may lead to death of neurons and oligodendroglial cells. These events include: release of oxygen free radicals; influx of calcium which activates phospholipase and causes membrane destruction; and high extracellular concentrations of excitotoxic amino acids with overstimulation of *N*-methyl-D-aspartate (NMDA) and other glutamate receptors. These processes are fully described in Chapter 17. Calcium channel blocking agents have been used clinically as vasodilators and might be therapeutic following severe hypoxia–ischemia by reducing calcium influx, dilating cerebral vessels, and possibly by reducing platelet aggregation and improving cardiac function. Steen *et al.*

(1983) demonstrated that the calcium channel blocker nimodipine given immediately after temporary aortic occlusion improved neurological recovery and cerebral blood flow in dogs.

Vaagenes and colleagues (1984) showed, in a controlled trial, that the calcium channel blocker lidoflazine given after 10 minutes of cardiac arrest in dogs produced significantly better neurological recovery, although histopathological scoring was not significantly different. Allen *et al.* (1983) showed that the calcium channel blocker nimodipine given to adult human patients with subarachnoid hemorrhage significantly reduced secondary cerebral vasospasm. A placebo-controlled, double-blind, randomized trial in 155 patients resuscitated after out-of-hospital ventricular fibrillation tested the effect of nimodipine injected immediately after restoration of spontaneous circulation (Roine *et al.* 1990). Overall, there was no difference in survival at one year (40% with nimodipine vs. 36% in the control group) but, in a post hoc analysis of patients with more than 10 minutes' delay before advanced life support, there was a higher rate of survival in the lidoflazine group (47% vs. 8%). There was no statistically significant difference between the two treatment groups in neurological outcome.

Levene and colleagues (1990) reported on the use of the calcium channel blocker nicardipine by continuous intravenous infusion in four severely asphyxiated human neonates. The mean arterial pressure fell in three out of four and there was a marked drop in arterial pressure with an associated fall in cerebral blood flow velocity in two infants. Avoidance of hypotension is one of the few principles of treatment of HIE that everyone agrees on, and calcium channel blockers have never been assessed in a large randomized trial in human newborns with asphyxia or HIE. Despite the positive results from animal models, there is doubt as to whether calcium channel blockers are safe or effective in protecting the brain after hypoxia. The timescale of calcium influx may be such that very early administration of a calcium channel blocker is necessary to have any therapeutic effect. In practice, very early administration within a few minutes is usually not possible in perinatal asphyxia.

Magnesium sulfate

Posthypoxic neuronal damage is partly caused by raised extracellular concentrations of the excitotoxic amino acids glutamate and aspartate, and the NMDA receptor is thought to be important in the pathogenesis of posthypoxic damage in the perinatal period. Increasing the extracellular concentration of magnesium can block the NMDA receptor by hyperpolarization. McDonald and colleagues (1990) were able to produce cerebral injury in a neonatal rat model by direct injection of NMDA. The extent of the cerebral damage was reduced by magnesium administration. Thordstein and colleagues (1993) treated 7-day-old rats with magnesium sulfate, mannitol and L-methionine after unilateral carotid ligation and two hours of hypoxia and demonstrated a significant reduction in cerebral damage. There is, however, no neonatal model of cerebral hypoxia–ischemia where magnesium alone has reduced the extent of brain damage. Magnesium sulfate had been widely used in obstetric practice for both preeclampsia and as tocolysis in preterm labor. Observational studies have reported an association between maternal magnesium sulfate treatment and a reduction in the rate of cerebral palsy in infants born prematurely (Hirtz & Nelson, 1998).

In 1993 it was suggested that clinical studies of magnesium sulfate as cerebral protection after severe birth asphyxia should begin in Europe. It had been well known for many years that high concentrations of magnesium could produce hypotonia, vasodilation, and muscle paralysis. The phase 1 study was to investigate the pharmacokinetics of magnesium and to examine the effects of two different doses of magnesium on blood pressure, heart rate, respiration, and neurological function (Levene *et al.*, 1995). Infants were eligible if: there was evidence of fetal distress; the gestational age was 35 weeks or more; Apgar score was lower than 6 at ten minutes; and postnatal age was less than 12 hours. Seven infants received 400 mg/kg of $MgSO_4 \cdot 7H_2O$. (Magnesium sulfate is normally in the heptahydrate form.) Eight infants received 250 mg/kg of $MgSO_4 \cdot 7H_2O$. The serum Mg^{2+} rose from 0.79 mmol/l to 3.6 mmol/l at one hour and was still elevated at 24 hours. Mean arterial pressure fell in the first hour by an average of 6 mmHg and in the most seriously ill infant, already requiring an infusion of dopamine at 12 μg/kg per minute, the mean arterial pressure fell from 40 mmHg to 25 mmHg five minutes after the magnesium sulfate infusion was completed. After increasing the dopamine infusion to 20 μg/kg per minute, the mean arterial pressure rose to 43 mmHg by 60 minutes. Mean heart rate fell by 10 beats/min but this did not reach statistical significance. All the infants receiving

400 mg/kg were already intubated and ventilated and ceased to breathe spontaneously for three to six hours after the magnesium infusion. Muscle activity and tone diminished but the EEG did not change.

The infants who received 250 mg/kg of $MgSO_4 \cdot 7H_2O$ showed a rise in serum concentration from 0.71 mmol/l to 2.42 mmol/l. There was no significant change in heart rate, blood pressure, muscle tone, or EEG, but one infant who was not initially intubated became apneic and required ventilation by face mask for five minutes. In two infants, cerebrospinal fluid concentrations of Mg^{2+} were measured and found to be lower than the corresponding serum level (1.43 vs. 2.15 mmol/l and 1.71 vs. 1.91 mmol/l). The experience from the phase 1 study and other anecdotal communications indicate that high doses of magnesium sulfate can produce potentially dangerous hypotension in asphyxiated infants. It is likely that the most severely asphyxiated infants, with hypoxic damage to the myocardium and endothelium, are the ones that are most vulnerable to cardiovascular collapse if a large dose of magnesium is administered. Two small randomized trials have tested magnesium sulfate in infants with HIE. Ichiba *et al.* (2002) randomized term infants with poor Apgar score and delayed respiration or later seizures to magnesium sulfate 250 mg/kg for 3 days or standard treatment. The infants treated with magnesium had a significantly more normal outcome (normal cranial CT, EEG, and established feeding by 14 days) (12/17) than the standard group (5/16). No serious adverse effects were seen. Bhat *et al.* (2009) randomized 43 term infants with perinatal asphyxia to magnesium sulphate 250 mg/kg for 3 days or normal saline for 3 days. At discharge 14/18 in the magnesium group had good outcome, compared to 7/18 in the placebo group. Thus magnesium sulfate looks promising but there is insufficient evidence on long-term outcome to recommend this intervention as standard of care. A large-scale randomized trial with long-term outcome is being planned.

Allopurinol

Reoxygenation after hypoxia–ischemia gives rise to free-radical-induced injury. Xanthine oxidase contributes to this by synthesizing hypoxanthine. Palmer and colleagues (1990) investigated the effect of allopurinol, a xanthine oxidase inhibitor, in the 7-day-old rat unilateral carotid ligation and hypoxia model. Allopurinol-treated rats had significantly less water in the ligated hemisphere at 42 hours and less severe neuropathological changes at 30 days.

Saugstad (1996) has pointed out that high activities of xanthine oxidase are restricted to a few organs (especially the liver) in humans. As positive effects from xanthine oxidase blockade with allopurinol have been reported even in organs containing relatively low concentrations of xanthine oxidase, allopurinol may exert its effect by either scavenging oxygen free radicals directly or augmenting adenine nuc1eotides. Another possibility considered was that hypoxic damage to the liver released large amounts of xanthine oxidase into the blood from where it could reach the brain. However, Rootwelt and colleagues (1995) showed that only low levels of xanthine oxidase were released into the blood after severe hypoxemia in newborn pigs. In the light of all the promising preliminary findings, there is considerable interest in a large randomized trial of allopurinol in infants with severe asphyxia or early HIE.

Van Bel and colleagues (1998) have studied the effect of high-dose allopurinol, an inhibitor of xanthine oxidase and a free radical scavenger, in severely asphyxiated newborn infants. No toxic effects from allopurinol were observed. Nonprotein-bound iron (a source of free radicals) rose in the control group but dropped to zero in the allopurinol-treated group. Malondialdehyde (an indicator of lipid peroxidation) increased in the control group but remained stable in the allopurinol group. Electrocortical brain activity (recorded by the CFM) decreased in the control group but remained stable in the allopurinol group during the first eight hours. Van Bel's group randomized 32 term infants with HIE to receive 40 mg/kg allopurinol within four hours, repeated 12 hours later. Thirteen of 17 (76%) died in the allopurinol group and 10/15 (67%) in the control group. Five of six had abnormal MRI findings in the basal ganglia or subcortex in each treatment group (Benders *et al.*, 2006). The authors concluded that allopurinol was given too late and they have now started a trial of allopurinol administration to mothers with evidence of fetal distress.

Hypothermia

Hypothermia at the time of hypoxia–ischemia has been well known for many years to protect the brain. This has enabled open heart surgery to be carried out and there have been numerous reports of near drownings in cold water where individuals have been

submerged for up to 40 minutes in cold water with complete recovery.

Early therapeutic neonatal use of hypothermia

Cooling was first reported as treatment for birth asphyxia by the Swedish obstetrician Bjorn Westin (1959). He reported six neonates who failed to respond to other forms of resuscitation and were placed in a bath of cold water so that colonic temperature fell to between 23°C and 30°C. The cooling began between four and 15 minutes after birth and was continued for up to 39 minutes. Only one of the six infants died. Similar reports have been made by Miller and colleagues (1964) and Cordey and colleagues (1973). It is important to point out that rapid cooling as a means of resuscitation of the newborn preceded the introduction of endotracheal intubation and ventilation. When effective oxygenation could rapidly be achieved by intubation and many doctors were trained to intubate in Western countries, interest in cooling evaporated after Silverman and colleagues (1958) reported that a cooler thermal environment for a number of days increased mortality in very low birthweight infants. Only in the Soviet Union was rapid cooling used in the resuscitation of asphyxiated neonates during the 1970s and 1980s. During this period, one of the cardinal principles of neonatal intensive care was the maintenance of normothermia throughout illness and procedure.

Therapeutic posthypoxic hypothermia in animal models

In 1989, Busto and colleagues reported that mild cooling after cerebral ischemia reduced the extent of brain damage in the CAl region of the adult rat hippocampus. In 1992, Carroll and Beek confirmed this finding and showed a dose–response effect. The earlier the cooling started and the longer the duration of cooling, the better the cerebral protection.

The first report showing cerebral protection in a neonatal animal model with posthypoxic hypothermia was by Thoresen and colleagues (1995). Twelve hours of mild hypothermia (four degrees reduction) prevented secondary energy failure in the newborn piglet, as determined by MRS. In 1996 Thoresen showed that three hours of posthypoxic hypothermia (six degrees reduction) was protective in the 7-day-old rat unilateral carotid ligation and hypoxia model as demonstrated by quantitative neuropathology at seven days (Thoresen *et al.*, 1996b). This therapeutic effect has later been shown to persist for six weeks (Bona *et al.*, 1998). Sirimanne and colleagues (1996) have shown that only two degrees of hypothermia lasting 72 hours reduced the extent of cerebral damage in the 21-day-old rat using unilateral carotid ligation and 15 minutes of 8% oxygen. Gunn and colleagues (1997) developed selective head cooling in the fetal lamb. Thirty minutes of carotid occlusion was followed, 90 minutes later, by cooling via a coil around the head for 72 hours. In a randomized study, 72 hours of cooling produced a large reduction in infarction and neuronal loss. A delay in starting cooling to 5.5 hours still gave protection (Gunn *et al.*, 1998a). If animals are stressed or not anesthetized while cooled, posthypoxic hypothermia is not protective (Thoresen *et al.*, 2001; Tooley *et al.*, 2003).

There is now considerable evidence in three species of newborn animal that posthypoxic cooling is protective, and that mild cooling by two to six degrees appears also to be safe. Posthypoxic hypothermia reduces apoptosis more than necrosis, reduces the free radical nitric oxide, reduces excitotoxic amino acids, and reduces the duration of seizures. On the basis of Gunn's research on fetal sheep, clinical trials have generally used 72 hours of cooling with a maximum delay of 5.5–6 hours.

Clinical trials of posthypoxic hypothermia in newborn infants

Gunn and colleagues (1998b) pioneered head cooling via a coil of tubing wrapped around the head. Cold water is pumped through the tubing, the water temperature being adjusted to the rectal temperature. A head cooling cap system made by Olympic Medical of Seattle was piloted in Auckland, London, and Bristol in 1998. Although described as "selective head cooling," it is in fact cooling via the head to achieve systemic cooling to a rectal temperature of 34–35°C. This treatment system was used for 72 hours in term infants who had clinical evidence of perinatal asphyxia, abnormal neurological signs, and abnormal aEEG within the first six hours. Figure 18.8 shows an infant with HIE being treated with the Olympic CoolCap system in Bristol. Pilot studies showed that heart rate fell (to 80–100 beats/min) and blood pressure rose by about 5 mmHg as the temperature fell with the reverse happening during rewarming (Thoresen & Whitelaw, 2000). Elimination of drugs by liver metabolism (e.g., phenobarbital) is prolonged by therapeutic hypothermia (Thoresen *et al.*, 2003).

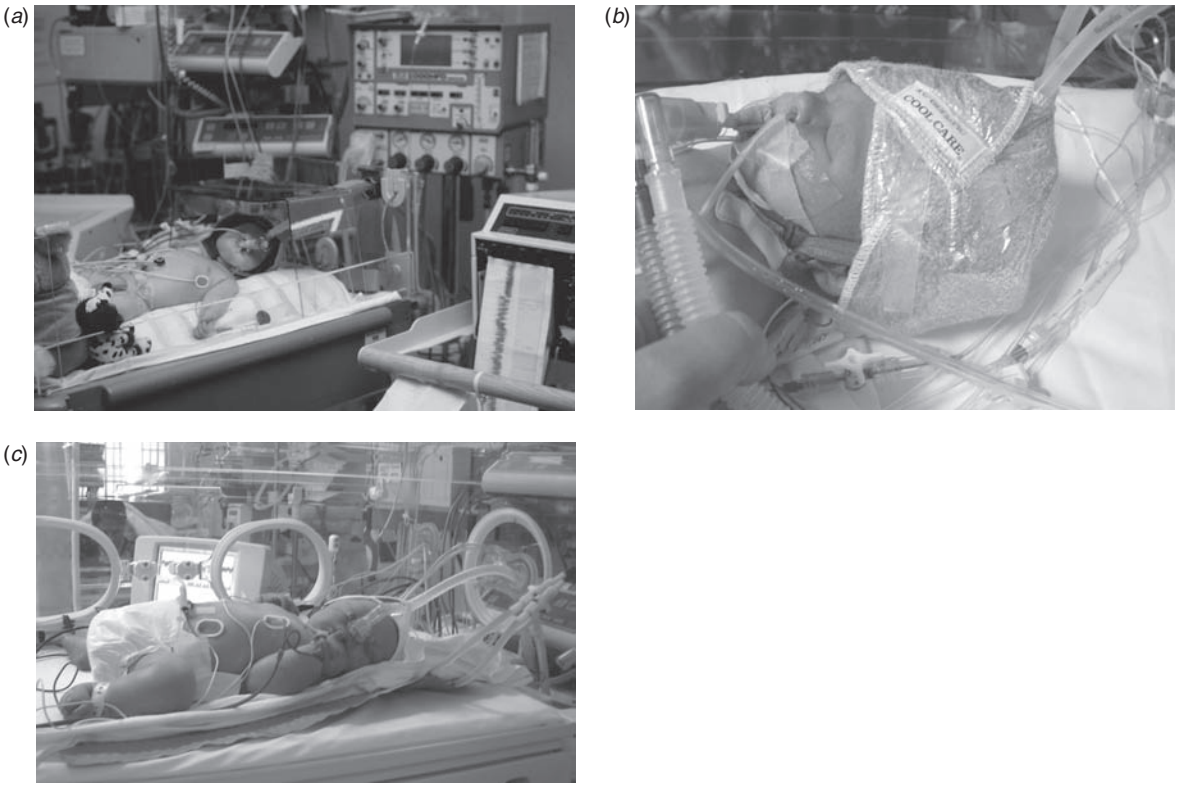

Fig. 18.8 (*a*) An infant with severe hypoxemic–ischemic encephalopathy (HIE) in the CoolCap Trial. The baby is intubated and ventilated. The CoolCap is on the head and there is a reflecting shield over the head. The aEEG is visible on the cerebral function monitor at the side of the cot. (*b*) Close-up showing the CoolCap with two water hoses attached. (*c*) A ventilated baby with HIE lying on a cooling mattress under the sheet and receiving whole-body hypothermia.

Randomized clinical trials of posthypoxic hypothermia in infants with HIE

Eicher and colleagues (2005) carried out a small randomized trial at six hospitals in 65 infants of 35 or more weeks with abnormal neurological signs together with two of the following: fetal heart rate <80 for 15 minutes, pH <7.1, base deficit >13, Apgar score <6 at ten minutes and postnatal saturation <70% for 20 minutes. Within six hours, infants were randomized to either a cooling blanket to a rectal temperature of 33°C for 48 hours or normothermia (37°C); 17% were lost to follow-up, 84% of the normothermic infants with known outcome died or were disabled at 12 months, but only 52% of the cooled infants were died or were disabled.

The first large multicenter trial of hypothermia was the CoolCap Trial (Gluckman *et al.*, 2005). Infants with gestational age 36 or more weeks were eligible if they had one of the following: pH <7.0; base deficit 16; and Apgar score <6 or need for resuscitation at 10 minutes. Abnormal neurological signs and abnormal aEEG were also required and the time limit for randomization was 5.5 hours. Infants were allocated to either selective head cooling with mild systemic hypothermia (rectal 34.5°C) for 72 hours or normothermia. Of 234 infants recruited in 25 centers in the United States, the United Kingdom, New Zealand, and Canada, 218 were followed up. Of these, 66% of the normothermic infants died or were disabled at 18 months but only 55% of the hypothermic infants died or were disabled. This study showed that hypothermia changes the prognostic value of clinical evaluation of neonatal encephalopathy. After 72 hours of hypothermia, the neurological examination can look more depressed than it would with normothermia, in infants who ultimately recover (Gunn *et al.*, 2008). This may be due to stiffness of edematous tissue and the delayed elimination of sedative and anticonvulsant drugs.

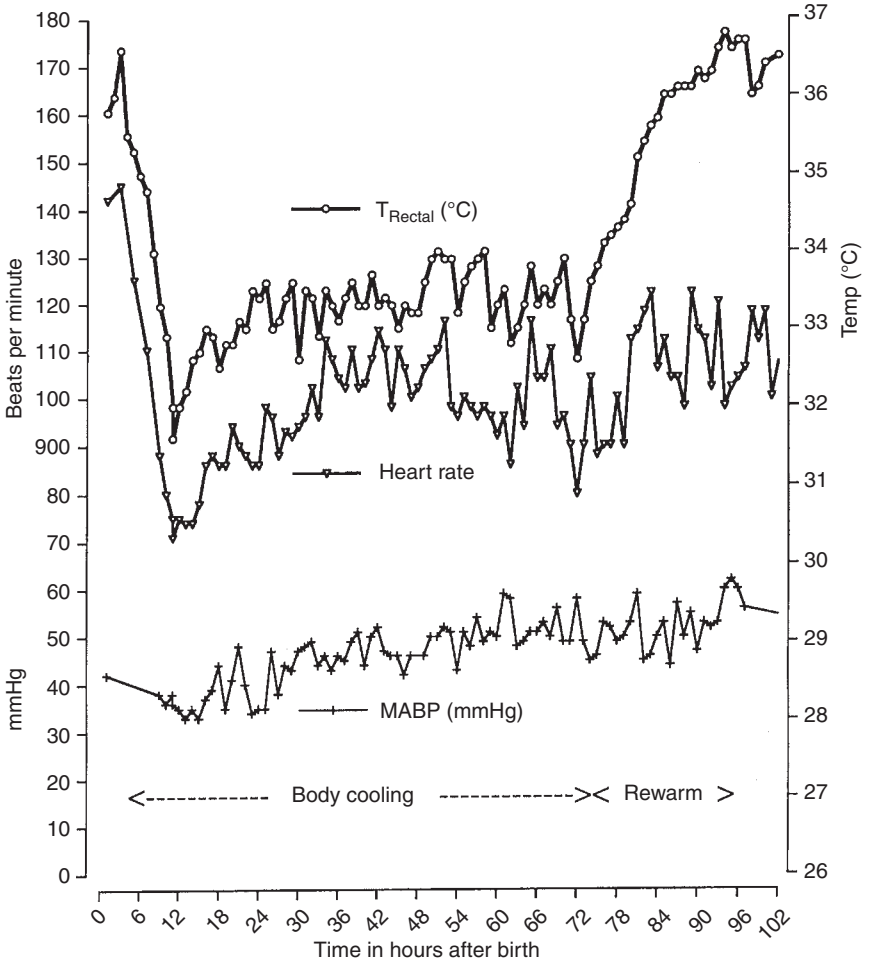

Fig. 18.9 Rectal temperature, heart rate, and mean arterial blood pressure (MABP) from an asphyxiated infant during 72 hours of mild systemic hypothermia.

The second large multicenter was conducted by the U.S. National Institute of Child Health and Development's Neonatal Network (Shankaran *et al.*, 2005). A total of 208 infants were recruited in 12 centers. Eligibility criteria were: pH <7.0 or base deficit 16, or an acute obstetric event as well as Apgar <6 or need for resuscitation at 10 minutes; and clinical signs of encephalopathy. Treatment allocation was to either systemic hypothermia at 33.5°C for 72 hours using a cooling blanket system or normothermia at 37°C; 62% of the normothermic infants died or were disabled at 18 months but only 44% of the hypothermic infants died or were disabled.

The third randomized clinical trial of hypothermia in neonatal HIE, the TOBY trial, is the largest, with 325 infants recruited. This used exactly the same entry criteria as the CoolCap Trial but the treatment was total body hypothermia at 33.5°C for 72 hours (Fig. 18.9). Cooled infants had increased rates of survival without neurological abnormality, neuromotor impairment, or cerebral palsy (Azzopardi *et al.*, 2009).

Eleven years of neonatal experience with therapeutic hypothermia has shown no serious adverse effects of cooling as long as appropriate staff and monitoring are in place. The clinical trials evidence is now sufficient to state that hypothermia is an effective and safe treatment for HIE in newborn infants at term. Therapeutic cooling has been standard of care in Bristol since the end of 2006 and is being introduced in neonatal intensive care units in many countries. There is no conclusive evidence as whether selective head cooling or whole-body hypothermia is better, but an MRI comparison of the two methods

of cooling found that both reduced basal ganglia and thalamic injury, although selective head cooling was better at preventing cortical injury (Rutherford *et al.*, 2005).

A number of research groups are currently investigating whether hypothermia combined with various pharmacological agents augments the protection obtained from hypothermia alone. There is evidence that inhaled xenon can safely increase the protection from hypothermia (Hobbs *et al.*, in press).

Current recommendations for clinical management of neonatal HIE

Maintenance of adequate ventilation and oxygenation after birth

In the current state of knowledge this means achieving an arterial pO_2 of 8–12 kPa (60–90 mmHg) with hemoglobin saturation 93% or more. There is no evidence that hyperoxia is beneficial and, in theory, it might be toxic. Saugstad (2005) has presented evidence that air is as effective as 100% oxygen in the resuscitation of asphyxiated newborns and may have advantages. If mechanical ventilation is necessary, it is important to avoid hypocapnia.

Hypocapnia reduces cerebral blood flow as well as altering oxygen delivery and possibly affecting a number of important pH-dependent enzymes. We are willing to permit mild hypercapnia during mechanical ventilation with $paCO_2$ up to 7.0 kPa (53 mmHg) as long as arterial pH is above 7.2. In addition to the considerable evidence linking hypocapnia with cerebral injury in preterm infants, there is an association between duration of hyperventilation and worse neurological outcome in full-term infants (Bifano & Pfannenstiel, 1988). Hypothermia affects the pCO_2. A 3.5°C reduction in temperature reduces pCO_2 by about 4 mmHg. If blood gases are measured at 37°C , one should then raise the target range of pCO_2.

Maintenance of adequate cerebral perfusion

The lower limits of the accepted range for mean arterial pressure in the full-term infant is about 40 mmHg (Versmold *et al.*, 1981*)* and this figure is widely used as an indication for intervention. In many cases, the clinician will not be able to exclude hypovolemia and a rapid infusion of 10–15 ml/kg of 0.9% sodium chloride is indicated. Echocardiography is increasingly used by neonatologists to assess filling of the heart and myocardial contractility. If there is strong suspicion of blood loss or anemia, then whole blood or red cells would be preferable. Despite being traditional as volume replacement for many years in neonatal medicine, albumin is not indicated and may increase the chances of fluid retention (So *et al.*, 1997). If volume replacement does not achieve adequate blood pressure, dopamine by continuous infusion is used as the first inotropic agent. If this proves inadequate, dobutamine may be added for more inotropic effect or norepinephrine for more peripheral vasoconstriction. Avoiding hypoxemia and circulatory impairment also involves avoiding unnecessary manipulations particularly of the airway, head or neck, and also minimizing pain and compression.

Maintenance of adequate blood glucose levels

After a severe hypoxic–ischemic insult, mitochondrial function may be disturbed for many hours despite reoxygenation and cells may be dependent on cytosolic glycolysis for production of energy in the form of adenosine triphosphate (ATP). Glycolysis produces fewer molecules of ATP from one molecule of glucose than does oxidative phosphorylation in the mitochondria. Thus glucose stores may easily become depleted. Hattori and Wasterlain (1990) showed that cerebral hypoxia–ischemia in the 7-day-old rat reduced brain glucose to 0.3 mmol/l. In a controlled study, these investigators showed that administration of glucose after hypoxia reduced the severity of neuropathological injury. In order to achieve a brain glucose of 3–5 mmol/l we now recommend maintaining blood glucose between 4 mmol/l and 6 mmol/l (75–100 mg/dl) in infants developing HIE.

Hypothermia in clinical practice

On the basis of all the evidence listed above, we do not actively warm asphyxiated term infants during resuscitation. Prolonged resuscitation will then usually result in body temperature dropping. Rectal temperature should be monitored. If neurological assessment and aEEG show evidence of encephalopathy, we then maintain a rectal temperature of 33.5°C for 72 hours using any of the available cooling methods. If neurological assessment and aEEG are both normal in the first six hours, we slowly re-warm the infant to 37°C.

We inform and discuss treatment with parents on an ongoing basis and have often told the parents the results of the published trials. No parent has refused hypothermia as treatment.

Avoidance of hyperviscosity

Polycythemia and hyperviscosity are not uncommon in fetuses with intrauterine growth retardation who are at increased risk of intrapartum asphyxia. Polycythemia is associated with lower IQ scores at school (Delaney-Black *et al*., 1989). It is still not clear how much difference dilutional exchange transfusion makes to outcome in newborn infants but the general recommendation that a venous hematocrit of 70% should be reduced even in the absence of symptoms should probably apply to an infant symptomatic with early HIE. It is relevant here to mention that hemodilution was introduced into acute stroke therapy in adults on the basis of animal modeling. Lee and colleagues (1994) showed in dogs with left middle cerebral artery occlusion that a 30% hematocrit gave a smaller infarct size than did hematocrit values of 40%, 35%, or 25%.

Control of clinical seizures

As mentioned in the section on barbiturate therapy, it is unclear whether subclinical electrical seizures in the newborn are damaging but there is agreement that repeated clinical seizures are likely, on balance, to be harmful. Clearly, seizures which impair respiration or circulation are potentially harmful. When seizure activity is not interfering with vital functions, it seems sensible that anticonvulsant therapy be started if a clinical seizure lasts three minutes or three briefer seizures occur within one hour (Levene, 1993).

Fluid restriction

Fluid volume is often restricted for infants with severe asphyxia but this is only really relevant (i) if the infant develops renal failure where a regimen of replacing urine output + insensible loss would be indicated or (ii) where there is evidence of inappropriate antidiuretic hormone. Fluid restriction has no role in prevention of brain injury and, indeed, maintenance of perfusion and blood glucose may necessitate short periods of increased fluid administration. Also, infants kept hypothermic tend to shift intravascular volume to the interstitial space and intravascular volume depletes. Cardiac assessment can diagnose this and guide treatment with volume.

Criteria for withdrawal of life support

An important part of the assessment and treatment of infants with severe HIE is the question of when withdrawal of life support is the best option. If the infant is in stage III (severe HIE) for 72 hours, and the clinical picture is supported by an EEG showing a low-amplitude or burst suppression pattern and low resistance index on cerebral Doppler, then the follow-up experience suggests that the chances of surviving to achieve independent existence with education, mobility, and employment are vanishingly low. In our view, withdrawal of life support should be discussed between the clinical medical and nursing staff responsible for the infant after 48–72 hours of severe HIE. If no reversible factors emerge and no improvement has occurred by this time then we have recommended to the infant's parents that life support is no longer in the child's best interests. In our practice, it is not necessary for the infant to be clinically brain dead to make this recommendation nor is it necessary to demonstrate total absence of cerebral blood flow or isoelectric (isopotential) EEG. The recommendation is based on the very poor quality of life if survival occurs. To withdraw life support is such a serious decision that it is essential that all the clinical team and the parents agree before support is actually withdrawn (Whitelaw, 1986). The discussions and the agreement should be noted in writing. Because the infant has been in a critical condition from early on, it will have been necessary for the neonatologist to have talked with one or both parents repeatedly during the first three days informing them of the evidence for brain injury, the treatment being given, and the methods used to assess the infant's response. Thus the discussion on withdrawal of treatment is a natural consequence from what has been talked about before. Leaving the decision to withdraw for longer than 72 hours may be, in our view, counterproductive. Thus we would not delay the decision while waiting for an MRI examination to be arranged (after a holiday weekend, for example). Even severely damaged infants will usually eventually breathe spontaneously and the longer one postpones extubation of an infant with HIE, the more likely it becomes that the baby will breathe or gasp spontaneously and survive. This is likely to be stressful if the baby is conscious at all and is very distressing for the parents and staff who have reluctantly come round to the view that the most humane thing is for the baby to die quickly and peacefully. In some cases, an unresponsive baby and

very-low-amplitude EEG will prompt discussion of withdrawal of treatment by 24 hours. In such cases, it is essential that all relevant diagnostic measures have been taken (to rule out other diagnoses, especially treatable conditions) and there has been enough time to see if there is a response to treatment.

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Chapter 19

Clinical aspects of brain injury in the preterm infant

Michael Weindling

Introduction

After about 32 weeks' gestation, the neurodevelopmental outcome of premature infants appears to be independent of gestation. However, before 32 weeks there is an almost linear relationship between IQ measured later in life and the gestation at which the baby was born. This was shown by a study of the neurodevelopmental outcome of preterm infants by Wolke and colleagues (2001). The authors assessed IQ at 4 years and 8 months. They considered the relationship between medical and social risk factors using data from the Bavarian Longitudinal Study, which had investigated the cognitive and behavioral development of children considered to be vulnerable because of neonatal adversity. Their conclusion was that cognitive and school outcome for infants born before 32 weeks' gestation was better predicted by neonatal risk (by which they meant prematurity and low birthweight) than social factors. The reverse was true for more mature infants. These data fit well with more recent information from very immature infants born before 26 weeks. A UK cohort of very premature babies born in 2000 were included in the Epicure study (see below), which looked at their outcome when they were 6 years old (Marlow *et al.*, 2005). The data from Bavaria and the UK have been combined in Fig. 19.1.

Thus, if birth occurs before about 32 weeks, the more premature an individual is, the greater the degree of disability. It also appears that babies born after 32 weeks, i.e., still about eight weeks prematurely, do not share this risk and are no more vulnerable than an infant born at term. This fascinating observation raises the intriguing question of what it is about the brains of very immature infants that causes their very considerable vulnerability, and why these factors should change with increasing maturity.

The focus of this chapter on clinical aspects of brain injury in the preterm infant is therefore the baby born before 32 weeks by postconceptional age (PCA). It considers factors affecting the growth of the developing brain for the critical period between about 20 weeks' and 32 weeks' gestation and two acquired causes of brain injury in the preterm infant: periventricular hemorrhage (PVH) and periventricular leukomalacia (PVL) or white matter injury (WMI) (Fig. 19.2). It also attempts to reflect recent developments in our understanding of the clinical problems that very immature infants have been found to have during later life and the ethical issues faced by those caring for these infants born at the "margins of viability" (Nuffield Council on Bioethics, 2006).

Several factors have affected our knowledge base. First, the survival of very immature infants has improved because of the widespread introduction of two technologies in the mid 1990s: antenatal steroids administered to mothers at risk of delivering prematurely and the use of artificial surfactant. Both interventions have independently doubled survival. Furthermore, their combined use has significantly decreased overall mortality compared with the effect of either treatment alone (Jobe *et al.*, 1993). Second, there have now been substantial follow-up studies. These have given us a much clearer idea of the outcome for infants born very prematurely. Third, there have been enormous advances in magnetic resonance imaging (MRI), which has shown us that the brains of these infants when they reach term equivalence are rather different from those of babies who were born at term after an uncomplicated pregnancy (see Chapter 13).

Clinical considerations

North American literature has coined the term "extremely low gestational age neonates" (ELGAN).

The Newborn Brain: Neuroscience and Clinical Applications, 2nd edn., eds. Hugo Lagercrantz, M. A. Hanson, Laura R. Ment, and Donald M. Peebles. Published by Cambridge University Press.

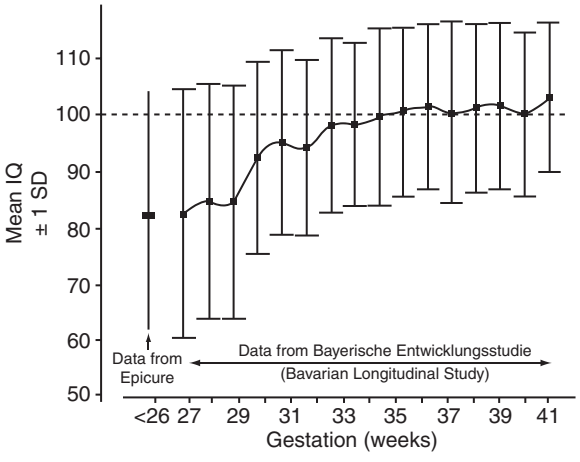

Fig. 19.1 IQ of babies born at various gestations. measured in two separate studies. The data from the Bavarian Longitudinal Study were collected when the children were 4 years and 8 months old (Wolke *et al*., 2001). The Epicure data were collected when the children were 6 years old (Marlow *et al*., 2005). (After an idea by N. Marlow.)

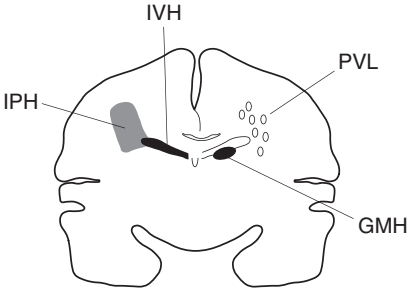

Fig. 19.2 Coronal section of the immature brain showing the sites of germinal matrix (GMH), intraventricular (IVH), and intraparenchymal (IPH) hemorrhages, and periventricular leukomalacia (PVL).

It emphasizes the unusual nature of these extremely small babies, who have only completed about 60% of their gestation and who only weigh about one-sixth of their weight at term. Their physical and neurological immaturity is striking. Their eyelids are still fused, the membrane between the upper and lower lids only breaking down after about 26 weeks by postmenstrual age. Their skin is thin and highly permeable to water. They make breathing movements (as they did in the womb), but these are ineffective. Before about 26 weeks, gas exchange in the lungs is not at the alveoli, which are still unformed, but through the terminal bronchioles. Sucking, a complex and integrated sequence of movements, is also incoordinated. In the more mature baby, the muscle groups of the tongue contract in such a coordinated fashion that they effectively strip milk from the nipple. Then, as the epiglottis closes to protect the airway, the tongue moves the globule of milk through the larynx to the oesophagus, a mechanism that only becomes effective after about 34 weeks. The situation was summarized elegantly by Vohr and Allen (2005):

> Infants born at between 22 [and] 25 weeks of gestation are fragile and vulnerable, with immature organ systems. … They are at high risk for brain injury from hypoxia and ischemia and undernutrition, as well as for sepsis, which starts the cascade of events that increase the risk of brain hemorrhage, white-matter injury (periventricular leukomalacia and ventriculomegaly), and poor brain growth, and for subsequent neurodevelopmental impairment. Active brain development occurs during the second and third trimesters, with neurogenesis, neuronal migration, maturation, apoptosis, and synaptogenesis. The immaturity of the brain at 22 to 25 weeks of gestation makes these extremely preterm infants particularly vulnerable.

The earliest gestation at which survival is possible is from about 23 weeks onwards. It is relevant to this discussion to note that neuronal migration is completed only a few weeks previously. This is considered in more detail in Chapter 4, but is probably at around 18–20 weeks postconception, although the exact time in the human is not known. Thus, at the time of their birth, the brain development of these very immature babies is at a critical stage because, with neurons at their final positions, the delicate process of synaptogenesis is taking place (Bourgeois, 1997) and the cellular milieu is therefore likely to be critical. However, the intrauterine environment, from which these babies emerge, is very different from that in which they find themselves after birth. Infants at, or close to, term are adapted to cope with this change, but very immature individuals are not. Current guidelines of the American Academy of Pediatrics advise neonatologists to warn parents that it is inappropriate to resuscitate infants of less than 23 weeks of gestational age or those whose birthweight is less than 400 g because of their very poor prognosis (Braner *et al*., 2000).

The cerebral circulation: anatomy and physiology

Anatomy

Some features of the embryology of the developing brain, described in detail by Pape and Wigglesworth (1979), are relevant to the present discussion. The basic pattern of the external cerebral vessels (the

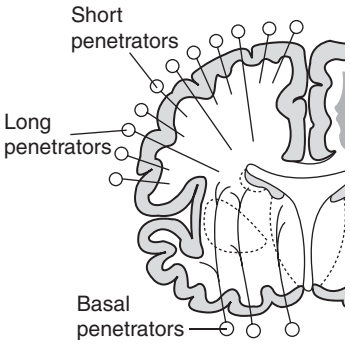

Fig. 19.3 Coronal section of cerebrum showing the long and short penetrating arteries, which supply the cerebral white matter. (Adapted from Volpe, 2001.)

internal carotid artery and branches of the anterior, middle, and posterior cerebral arteries) is established by 7 weeks' postconceptional age, and the postembryonic period is characterized by the growth and development of the cerebral hemispheres. This is reflected by the pattern of development of the internal cerebral vessels. Blood vessels first form because of sprouting of vessels in the pial plexus around the neural tube (Collins, 1995). These sprouts then extend between the ventricular and marginal zones. Meningeal perforating branches pass into the brain parenchyma as cortical, medullary and striate branches. Short cortical branches supply a peripheral part of the cerebrum and long penetrating vessels supply more central regions (Fig. 19.3).

Neuronal precursors proliferate and migrate towards the pial surface of the brain. Glial cell processes, which stretch radially across the full thickness of the wall of the telencephalon and provide contact guide paths, guide the peripheral migration of these neurons. Deep progenitor cells then migrate to the superficial layers with the oldest cells taking up the deepest positions and being bypassed, or "leapfrogged," by younger cells, the youngest making up the outer layer of the neocortex. The processes of proliferation, neurogenesis, and gliogenesis are not necessarily sequential (Dobbing & Sands, 1970). An important consequence of this process, which is relevant to the development of germinal matrix hemorrhage–intraventricular hemorrhage (GMH–IVH), is the relative, but temporary, prominence of the subependymal germinal matrix between the postembryonic period and about 30 weeks' postconception. ("Germinal matrix" is a term favored by clinicians, although the Boulder Committee [1970] preferred to call the region the "ventricular zone" [Collins, 1995].)

The germinal matrix, situated in the floor of the lateral cerebral ventricles over the head and body of the caudate nucleus and in the notch between this nucleus and the thalamus, is packed with glioblasts, which take part in the migratory process (Pape & Wigglesworth, 1979). After about 30 weeks, the germinal matrix becomes progressively thinner. This metabolically active area is supplied by the recurrent artery of Heubner, a branch of the anterior cerebral artery that is prominent during this period of fetal life but which becomes relatively insignificant in the adult. The germinal matrix comprises an extensive capillary bed and is reckoned to be the source of about 80% of subependymal and intraventricular hemorrhages (Pape & Wigglesworth, 1979). Between 32 and 34 weeks' gestation the germinal matrix involutes and Heubner's artery eventually supplies just a small area at the head of the caudate nucleus (Collins, 1995). PVH becomes increasingly less common because of regression of this area.

As the cortex becomes more complex and its gyral pattern becomes more pronounced, the balance of the blood supply moves from the germinal matrix to the cortex. By the end of the third trimester, the blood supply has shifted from a circulation that is mainly central to one where most of the supply is to the cortex and subcortical white matter, a similar situation to that of the adult. The change in the position of the boundary or watershed areas explains the different distributions of ischemic brain lesions in babies. In the premature brain (i.e., before about 32 weeks' post conception) the periventricular white matter in the watershed between the centripetal and centrifugal arteries is particularly vulnerable. In the infant at or near to term the area of vulnerability has changed to the cortical and subcortical regions.

As development advances, there are also changes in the morphology of blood vessels. Before about 30 weeks, vessels in the germinal matrix are immature: there is no complex basal lamina or glial sheet, the endothelium is thinner than in cortical vessels and there is no smooth muscle, collagen, or elastic fibers, with relatively little reticulin (Collins, 1995). The lack of smooth muscle is likely to interfere with the ability of these vessels to change diameter and take part in autoregulation, and the lack of other

components may make them particularly vulnerable to changes in intraluminal pressure.

Physiology

Three features are relevant here: the relative resistance of the preterm brain to hypoxia; the ability to divert blood to essential organs (the diving reflex); and the concept of autoregulation.

Resistance to hypoxia

The fetus is hypoxemic compared with the baby after birth, the PaO_2 being about 20–25 mmHg (Teitel, 1998), but not hypoxic. The pH of fetal blood is between 0.1 and 0.15 units below the pH of maternal blood in late gestation, and the fetus is about 0.5°C warmer than the mother. These factors have the effect of shifting the oxygen dissociation curve to the right, facilitating the release of oxygen from the hemoglobin molecule to the fetal tissues. However, the high proportion of fetal hemoglobin (HbF), which is particularly prevalent at earlier gestations, ensures that fetal red cell oxygen affinity is greater than that of the maternal red blood cells. A hematocrit that is significantly higher in the fetus than after birth also has an effect in ensuring an adequate oxygen delivery to fetal tissues. The net effect of these factors is that fetal arterial blood oxygen content and oxygen saturation are similar to those of the human adult (Delivoria-Papadopoulos & McGowan, 1998).

The diving reflex

The ability to divert blood from the splanchnic circulation to the cerebral circulation is well established in term infants. However, the degree to which it operates in the extremely preterm infant remains uncertain, as is the degree to which it is altered by intrauterine growth restriction.

Autoregulation

Cerebral blood flow (CBF) is maintained, provided blood pressure remains within normal limits. These limits have not, however, been clearly defined in the preterm infant, and are likely to vary with other factors such as the availability of metabolic substrates, e.g., glucose (Pryds, 1994), and acidemia. Furthermore, CBF in the preterm infant seems to be particularly susceptible to hypocarbia, which would be expected to cause cerebral vasoconstriction, and the condition is associated with the development of PVL (see below).

Periventricular hemorrhage, periventricular leukomalacia, and white matter injury

Periventricular hemorrhage

In this chapter the term PVH is used to describe the conditions GMH or subependymal hemorrhage (SEH), IVH, and intraparenchymal hemorrhage (IPH) (Fig. 19.2). It is a disease of prematurity affecting about 40% of infants below 35 weeks' gestation or 1500 g and only very few babies above 37 weeks' gestation. The etiology of subependymal and intraventricular hemorrhages is rather different from that of intraparenchymal hemorrhages (see below). In its minor and commoner forms, when it affects only the germinal matrix or the cerebral ventricles, it has such little clinical effect that it is considered to be benign. It is only when the bleeding involves the adjacent brain parenchyma that there may be clinical effects. Because the condition is often unilateral, the clinical presentation usually takes the form of a hemiplegia – spasticity affecting one side of the body more than the other, and usually the arm more than the leg.

Germinal matrix bleeding usually arises over the caudate nucleus. Banker and Larroche (1977) found bleeding into the choroid plexus in 25% of autopsied neonates, but usually in addition to SEH and only to a small extent. In the more immature infants, the hemorrhage is over the body of the caudate nucleus, but, as gestation increases, bleeding becomes more likely to occur over the head (Leech & Kohnen, 1974; Pape & Wigglesworth, 1979). A great deal has been learnt from cranial ultrasound scanning about the timing and clinical associations of PVH. Germinal matrix and intraventricular hemorrhages become more common with increasing immaturity, and have been associated with respiratory distress and its complications (particularly pneumothorax) and asphyxia (e.g., Weindling *et al.*, 1985a). Although it has been noticed in up to 6% of stillbirths (Leech & Kohnen, 1974; Pape & Wigglesworth, 1979), PVH usually occurs within 48 hours after birth (between 40% and 50% occur within the first eight postnatal hours (e.g., Tsiantos *et al.*, 1974; Ment *et al.*, 1995). These observations suggest that

maturity of the vascular bed in the germinal matrix is important in determining whether bleeding occurs. Abnormalities of coagulation are not usually associated with PVH.

Parenchymal PVH is less common than GMH, but its clinical consequences are more serious. There is hemorrhagic necrosis in the periventricular white matter, usually just dorsal and lateral to the external angle of the cerebral lateral ventricles (Fig. 19.2) and usually asymmetrically (Volpe, 1998). The pathogenesis of intraparenchymal hemorrhage is rather different from that of minor germinal matrix and intraventricular hemorrhages. Here, two mechanisms may occur, probably separately, although they could coexist. Pape and Wigglesworth (1979) observed that massive germinal matrix hemorrhage was associated with congestion of the branches of the terminal vein that drains the area, and that the related white matter may become infarcted. Thus, venous infarction occurs after the drainage of deep veins has been obstructed, perhaps because of dilation of the lateral cerebral ventricles due to IVH or because of obstruction of the terminal vein in the subependyma (Pape & Wigglesworth, 1979; Volpe, 1998). Another possible explanation is that bleeding may occur into parenchyma that has been injured by ischemia, i.e., when the baby has been subjected to hypoxic ischemia around the time of birth (Weindling *et al.*, 1985a; Volpe, 1998).

The clinical associations between PVH and prematurity, hypoxic ischemia, and respiratory distress and its complications (pneumothorax, metabolic acidosis, and hypercarbia) have been known for a long time, and confirmed by clinicians using cranial ultrasound (for reviews, see Sinha *et al.*, 1985; Weindling *et al.*, 1985a, 1985b; Levene & de Vries, 1995). The interactions of these various factors are summarized in Fig. 19.4.

The clinical features of PVH largely depend on the size of the hemorrhage, and whether there is associated damage to brain tissue. At the time of a large bleed, the infant may collapse, either because of a sudden rise in intracranial hemorrhage or because of a fall in blood pressure or because of anemia. Papile and colleagues (1978), using computed tomography, found that the condition was undetected by the attending clinicians about half the time. In practice, a fall in hematocrit should lead to the suspicion that there may have been an intracranial disaster, but the diagnosis is readily made at the cotside using cranial ultrasound. However, since most PVHs are small and confined to the germinal matrix or within the cerebral ventricle, such a picture is unusual. When there is hemorrhagic infarction, an affected infant may be comatose or have seizures (notoriously difficult to detect and varied in manifestation in the premature neonate), or the bleeding may be entirely unnoticed by even the most vigilant and experienced clinical staff. Mostly infants are asymptomatic.

In the medium term, the main complication of PVH is posthemorrhagic hydrocephaly, particularly when there is hemorrhagic infarction of the brain parenchyma. A trial that looked at the effectiveness of draining intraventricular fluid compared with the use of diuretic therapy found no advantage to ventricular tapping (Ventriculomegaly trial, 1990; for a review, see Whitelaw *et al.*, 1996). More recently, there has been interest in fibrinolytic therapy: a clot-busting drug, streptokinase, was introduced into the ventricular system but there was no advantage when compared with conservative treatment (Whitelaw *et al.*, 1992; Hudgins *et al.*, 1994; Whitelaw *et al.*, 1996; Whitelaw & Odd, 2007). The incidence of posthemorrhagic hydrocephaly has, however, declined markedly over the past five years or so, probably because of the increased use of antenatal steroids (see below).

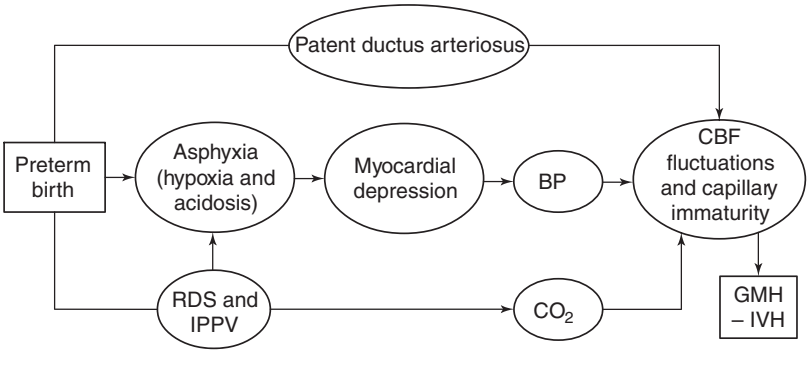

Fig. 19.4 Diagram of the pathways relating preterm birth to GMH–IVH. BP, blood pressure; CBF, cerebral blood flow; IPPV, intermittent positive pressure ventilation; RDS, respiratory distress syndrome.

Table 19.1 Affect of PVH on development assessed using the Bayley scales (Ross *et al.*, 1996)

	Preterm with minor PVH	Preterm without PVH	Term controls
Mental Development Index	102 ± 16	105 ± 19	109 ± 20
Psychomotor Development Index	98 ± 19	97 ± 16	106 ± 16

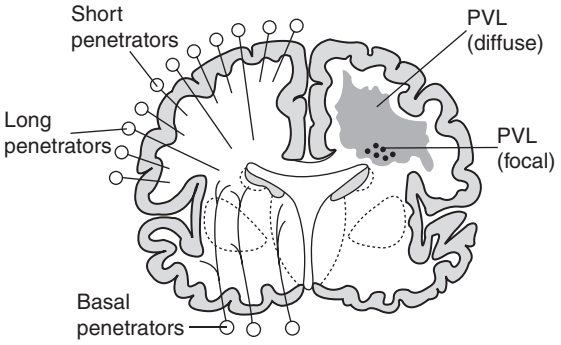

Fig. 19.5 Coronal section of cerebrum showing the cerebral vasculature in one hemisphere and focal and diffuse components of white matter injury (periventricular leukomalacia [PVL; diffuse] and PVL [focal]) in the other. The long and short penetrating arteries, which supply the cerebral white matter, are shown. The focal necrotic component of PVL is depicted by the black circles, and the diffuse oligodendrocyte-specific component in the gray shading. (Adapted from Volpe, 2001.)

The long-term complications of PVH mainly affect those infants in whom there has been white matter destruction, i.e., those with periventricular hemorrhagic infarction. About 5% of infants with isolated IVH later develop cerebral palsy (Levene & de Vries, 1995). Even when there is posthemorrhagic hydrocephaly, two separate studies, ten years apart, have shown that the pattern of disability was related to the associated brain damage, rather than to the process that had led to hydrocephaly (Cooke, 1987; Fletcher *et al.*, 1997). Children with subependymal and mild intraventricular hemorrhages have been found to perform only slightly less well on cognitive tests than gestation-matched controls without mild PVH or term infants (Table 19.1; Ross *et al.*, 1996).

In conclusion, PVH is a condition that affects premature infants, and its incidence increases with increasing prematurity. Minor degrees of PVH are relatively common, but are not associated with major neurodevelopmental disability. Severe PVH causes white matter destruction and consequent disability, and may lead to posthemorrhagic hydrocephaly. Fortunately, the incidences of intraparenchymal hemorrhage and posthemorrhagic hydrocephaly have declined over the past seven years or so. The decline occurred at about the same time – between 1990 and 1993 – as antenatal steroids and postnatal surfactant entered general clinical use.

White matter injury and periventricular leukomalacia

Our understanding of PVL, which affects the developing white matter, has changed with an appreciation that the condition is more widespread than previously thought. A better term is WMI, which may be focal or diffuse (Fig. 19.5) (Volpe, 2001). Although PVH and WMI generally affect the same group of infants, namely those born two or more months prematurely, the conditions are separate and have different etiologies and clinical effects. PVH is often asymmetric but WMI is usually generalized and bilateral, although not always evenly distributed, and therefore probably caused by a generalized brain injury.

It was previously thought that brain damage affecting the preterm infant born before 32 weeks' gestation mainly caused motor disability, usually manifest as spasticity (stiffness) of the legs more than the arms, a condition known as spastic diplegia. Sigmund Freud appropriately called it "spasticity of prematurity." However, follow-up studies and MRI have shown that WMI may have more diverse consequences, affecting cognition and complex processing.

WMI typically affects infants before 34 weeks' gestation. The peak timing of this condition means that it occurs at a time of white matter development before active myelination – microscopic myelin does not occur until after the first postnatal month, and myelin tubes only appear at between 11 and 13 months post conception (Back & Volpe, 1997).

Estimates of the prevalence of PVL vary. A general figure (the median of 13 studies between 1983 and 1992; for a review, see De Vries & Levene, 1995) is that it affects about 8% of infants below 32 weeks' gestation, and such infants comprise about 0.7% of all babies born. Ringelberg and van de Bor (1993) recorded an incidence of 5.4% in a hospital-based

study in the Netherlands, but an extraordinary geographically based study in northern Finland using MRI found PVL in 32% of premature infants, and not at all in a control group of children born at term (Olsén *et al.*, 1997).

PVL was first fully described by Banker and Larroche (1962), although Virchow described a similar condition in 1867. The basic gross appearance on sectioning the affected brain is of small white spots around the lateral cerebral ventricles, and this explains the condition's name (Greek λευκος = white, μαλαγια = softness). The following description of histopathological changes is based on Kinney and Back (1998) and Back *et al.* (1998). Between three and eight hours after injury starts, there is coagulation necrosis of all cells. Within 12 hours, astrocytes start to proliferate at the edge of the lesion and there is capillary hyperplasia. Between the next three and ten days, microglia start to infiltrate the damaged area, followed by reactive hypertrophic astrocytes and there is accumulation of lipid-laden cells. As the damaged area becomes organized, reactive gliosis occurs. Then, over the next few weeks, there is cavitation and/or gliosis. These show up on cranial ultrasound scans first as echodense areas around the ventricles. Then, between two and four weeks later, cavities, known as cysts, appear: the average time before cysts or cavities appear on ultrasound is three weeks (e.g., Weindling *et al.*, 1985a; Trounce *et al.*, 1986; Pierrat *et al.*, 2001). Because the condition invariably represents white matter damage, it is much more serious than PVH. Even extensive PVL is compatible with survival, and, typically, spasticity is not manifest until the baby is six or eight months old. Cognitive changes may only be recognized after several years and are considered in the section on outcome.

The pathogenesis of WMI is complex and not yet completely understood. In 1979, Pape and Wigglesworth described PVL as the most common ischemic lesion of the preterm brain, but the etiology is undoubtedly more complicated and WMI may affect some infants around term and even adults (e.g., Ginsberg *et al.*, 1976). Nevertheless, the distribution of the lesions, which are mainly in the periventricular watershed area, suggests that hypoxia and ischemia are important. Three factors have emerged. The first relates to hemodynamics and the striking observation that the lesions of PVL are in an area of the developing brain that is particularly vulnerable to ischemia. Furthermore, there is an association with hypocapnia (e.g., Calvert *et al.*, 1986; Greisen *et al.*, 1987). Since low carbon dioxide tension has a powerful cerebral vasoconstrictor effect (Kissack *et al.*, 2004), the link is plausible and ties in with the watershed distribution of focal WMI. Hypocarbia leads to cerebral vasoconstriction (and possible loss of the ability to autoregulate) and hence to hypoperfusion of the vulnerable periventricular region. A direct link with severe hypotension is less clear, although there is an association (summarized by Rennie, 1997). Using near infrared spectroscopy, low cerebral blood flow (4.9 ml/100 g per minute vs. 60 ml/100 g per minute in adults) has been recorded in low birthweight infants without apparent cerebral infarction (Tyszczuk *et al.*, 1998). The authors of that paper observed that their study did not support the assumption that a low mean arterial blood pressure (between 24 mmHg and 30 mmHg) was necessarily associated with damaging cerebral perfusion.

Another notable observation, which supports a notion that WMI is due to a perfusion abnormality, is that an increased risk of cerebral palsy (probably due to WMI) has been noted in monochorionic twins. The likely cause is altered intrauterine hemodynamics (Williams *et al.*, 1996; Pharoah & Cooke, 1997; Landy & Keith, 1998), based on the following argument. The crude rate of cerebral palsy per 1000 survivors at one year rises with increasing plurality. It is 1 or 2 in singletons, between 6.7 and 12.6 in twins, and between 28 and 44.8 in triplets (Grether *et al.*, 1993; Petterson *et al.*, 1993; Williams *et al.*, 1996; Pharoah & Cooke, 1996). The relative risk in normal birthweight twins is 4.5 and this is not explained by their increased risk of prematurity and low birthweight (Williams *et al.*, 1996). Thus, in a considerable proportion of singletons, spastic cerebral palsy may be due to the death of a monochorionic cotwin (Pharoah & Cooke, 1997). The mechanism is probably through the disruption of the supply of blood and oxygen to the surviving fetus, causing damage to vulnerable areas of the brain, for example in the watershed areas. Support for this hypothesis comes from the observation that cerebral palsy is more likely to affect a surviving twin if the cotwin died during the third trimester: Yoshida and Matayoshi (1990) observed that, when a monozygotic twin died early in pregnancy, the survivor survived without cerebral palsy. What probably happens is that blood flows from the survivor through artery-to-artery or vein-to-vein anastomoses into the lower resistance circulation of the dead monochorionic twin (Benirschke, 1993; Grafe, 1993). In a series

of 39 children with cerebral palsy resulting from 30 twin births, PVL was observed in 79% of the 24 babies who were born prematurely (Shimogaki *et al.*, 1998). Similarly, more children developed PVL among infants who had been exposed to multifetal pregnancy reduction than singletons (29% vs. 1.9%, odds ratio [OR] 21, 95% confidence interval [CI] 6 to 79) (Geva *et al.*, 1998). The same group also noted a significant excess of in vitro fertilization (IVF) and twinning among the infants who developed WMI.

Although a single causative organism has not been identified (Romero *et al.*, 1991), there is accumulating evidence that an association with chorioamnionitis, observed by Spinillo and colleagues (1995) among others, constitutes a probable mechanism. There is a persuasive argument (summarized by Dammann & Leviton, 1997) that the action of inflammation-related cytokines links chorioamnionitis with PVL. The evidence is circumstantial but a mechanism might be through neuropeptides and neuronal gene expression, which is also influenced by glucocorticoids (see below) (Patterson & Nawa, 1993). A rabbit model demonstrated that intrauterine infection can cause fetal brain white matter lesions (Yoon *et al.*, 1997). The same group found raised interleukin 6 (IL6) concentrations in umbilical cord plasma in infants who had PVL-associated lesions on early cranial ultrasound scan but a cord plasma IL6 concentration of ≥400 pg/l had only a positive predictive value of 14% for identifying neonates with cystic PVL (Yoon *et al.*, 1996). The nature of the inflammatory response appears to be important. In a mouse model, IL1β, IL6, IL9 or tumor necrosis factor (TNF) α (but not IL4) induced white matter lesions when injected with ibotenate, a glutamatergic agonist acting on *N*-methyl-D-aspartate (NMDA) receptors (Dommergues *et al.*, 2000). Hansen-Pupp and colleagues (2005) measured pro-inflammatory cytokine levels just after birth in infants with a mean gestation of 27 weeks. Premature rupture of membranes was associated with raised levels of IL2, interferon (IFN)γ and TNFα. Levels of IFN*γ* were high in infants developing WMI. Infants who were hypotensive had an increase in IL6 with a peak at six hours. Severe PVH was associated with increase in IL6 and IL8 concentrations (OR 2.8 and 13.2, respectively) and a fetal immune response with very high postnatal levels of IFNγ (OR 26.0) was associated with development of WMI.

Verma and colleagues (1997) reported that clinical chorioamnionitis doubled the chance of an abnormal cranial ultrasound scan during the neonatal period (OR 2.03, 95% CI 1.24 to 3.30). On metaanalysis, clinical chorioamnionitis and preterm delivery was significantly associated with both cerebral palsy (relative risk [RR] 1.9%, 95% CI 1.4 to 2.5) and cystic PVL (RR 3.0, 95% CI 2.2 to 4.0) (Wu & Colford, 2000). Since intrauterine infection causes premature labor (Goldenberg *et al.*, 2000), it is possible that the brain damage is a consequence of the infection, and that the time of premature birth affects the pattern of injury, rather than being its cause. There is also a suggestion that delivery by cesarian section might be helpful: Baud and colleagues (1998) reported that, in 99 preterm infants, 16 of whom developed cystic PVL, the risk of developing PVL was reduced significantly in those delivered by cesarian section (OR 0.15, 95% CI 0.04 to 0.57). This observation may be explained by the fact that antioxidant levels are higher in babies delivered by cesarian section than those delivered vaginally (see below and Georgeson *et al.*, 2002).

The vulnerability of neonatal cerebral white matter to injury before about 34 weeks' gestation may be explained by experiments using cell culture. The early differentiating oligodendrocyte is more susceptible to injury by oxygen-derived free radicals than the mature cell, possibly because of poorly developed antioxidant systems at this stage of development (Kinney & Back, 1998; Volpe, 1998). The observation that there is coagulation necrosis suggests that glutamate toxicity may play a part in perinatal WMI (summarized by Kinney & Back, 1998) (Fig. 19.6).

There are no specific clinical manifestations associated with WMI during the immediate neonatal period (e.g., Amiel-Tison, 1973; Pape & Wigglesworth, 1979). Abnormal neurological features occur after several months and vary according to the regions of the brain that have been damaged. Because the sites of periventricular infarction lie in the path of the motor tracts (Fig. 19.7), PVL generally causes motor disability, which takes the form of spasticity. This is usually described as a diplegia (when the legs are affected more severely than the arms), but when the lesions are widespread all limbs may be affected (spastic quadriplegia). The more of the brain that is affected by the lesions of PVL, the more severe will be the ensuing disability. Very extensive damage causes severe cognitive impairment, as well as motor disability. When the lesions are posterior, the optic radiation may be affected, resulting in cortical visual impairment,

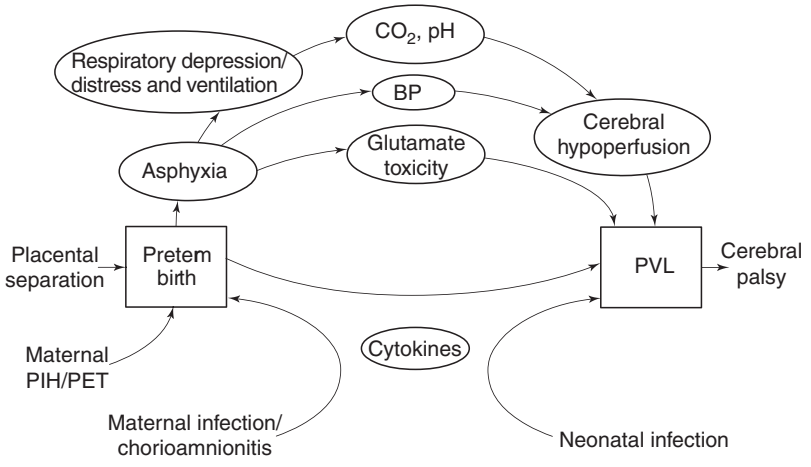

Fig. 19.6 Diagram of the pathways relating preterm birth with periventricular leukomalacia (PVL) and cerebral palsy. BP, blood pressure; PET, pre-eclamptic toxemia; PIH, pregnancy-induced hypertension.

sometimes with delayed visual maturation (e.g., Van den Hout *et al.*, 1998). Even when the ultrasound appearances have not progressed beyond the appearance of transient echodensities without cavitation, there is an increased incidence of minor motor disabilities (Ringelberg & van de Bor, 1993). In their study in northern Finland, Olsén and colleagues (1997), studying a group of 8-year-old children who had been born prematurely, found evidence on MRI of WMI in all children with cerebral palsy. There was also evidence of PVL in 25% of children with minor neurological dysfunction and in 25% of children born preterm but who were clinically healthy, but in none of those who had been born at term.

Spinillo and colleagues (2004) reported in an observational study that antenatal exposure to multiple antenatal courses of corticosteroids, particularly dexamethasone compared with betamethasone, was associated with the postnatal development of WMI. This raised concerns in view of reports of adverse effects of repeat doses of corticosteroids on neuronal myelination (Dunlop *et al.*, 1997) and brain weight in sheep (Huang *et al.*, 1999). However, this was not confirmed by Crowther and colleagues (2007), who studied children aged 2 years whose mothers had been given either multiple doses of antenatal corticosteroid (betamethasone) or a single dose seven or more days before premature birth at a median gestation of 28 weeks. There was no difference in neurodevelopmental outcome, including child behavior. However, as the authors pointed out, the children were relatively young and executive function could not be measured reliably (Crowther *et al.*, 2007).

In summary, WMI is the result of injury to the white matter around the lateral cerebral ventricles.

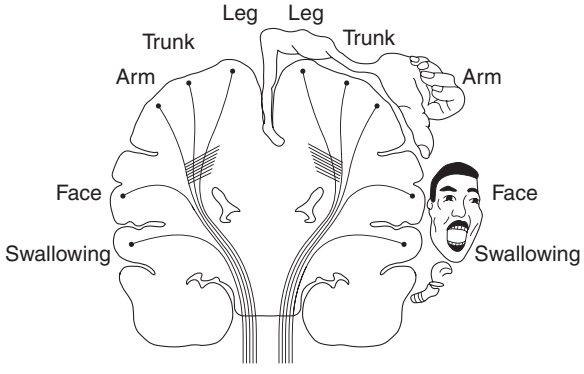

Fig. 19.7 Figure showing the (usually) symmetrical distribution of the lesions in periventricular leukomalacia (PVL) (hatched) affecting the lower, rather than the upper, limbs or face.

Chorioamnionitis, infection, hypocapnia and cerebral hypoperfusion, and immaturity are implicated in its etiology. It is likely that the disease represents the consequence of a process or sequence of events, which begins around the time of birth, sometimes before birth, but which is manifest afterwards. Even though cavities in the brain parenchyma ("cystic lesions") are clearly seen on ultrasound scans during the days and weeks just after birth, it is surprisingly difficult to make reliable predictions about outcome. In one intervention study where entry depended on the presence of signs of serious brain damage on an ultrasound brain scan, cerebral palsy was only accurately predicted in 54% infants (Weindling *et al.*, 1996). There are two possible reasons for this. One is that it is difficult to identify the precise anatomical location of the lesions. The other is that the damaging process occurs relatively early in the brain's development, and neuronal plasticity may compensate.

Neurodevelopmental outcome

Disability is common among extremely premature infants who survive. There have been several important and well-conducted follow-up studies, but this section concentrates on just a few and considers the outcome in childhood, adolescence, and young adulthood.

Survivors of low birthweight who have subtle cognitive abnormalities have been shown by brain MRI studies to have diminished volumes of the caudate nucleus and hippocampus, which are associated with lower IQ, learning disorder, and attention deficit disorder (Table 19.2; Abernethy *et al.*, 2003, 2004). Brain MRI studies of preterm infants also show that quantitative cerebral structural abnormalities are related to the degree of immaturity at birth (Inder *et al.*, 1999). Furthermore, preterm infants assessed at term show reduced myelination when compared with infants born at term (Inder *et al.*, 1999, 2005).

Childhood

A highly informative study relating to the outcome of very immature babies has been the UK EPICURE study (Costeloe *et al.*, 2000; Wood *et al.*, 2000). All children who were born at 25 or fewer completed weeks of gestation in the United Kingdom and Ireland during a ten-month period in 1995 were included. The study confirmed that they were a highly vulnerable group: only 39% survived. Of the survivors, 17% had parenchymal cysts and/or hydrocephalus (Costeloe *et al.*, 2000). One of the predictors of major brain damage seen on brain ultrasound scan was failure to administer antenatal steroids (see below). When these children were assessed at a median age of 30 months, severe disability was found to be common (Wood *et al.*, 2000). Using a population mean reference of 100, the mean ±SD scores on the Bayley Mental and Psychomotor Developmental Indexes were 84 ± 12 and 87 ± 13, respectively. The results for each gestational age group by weeks are shown in Fig. 19.8.

Table 19.2 Cerebral tissue volumes (mean ± SD) for premature babies at term-equivalence and term-born infants (Inder *et al.*, 2005).

Tissue class volume (ml)	Premature infants (n = 119)	Term-born infants (n = 21)	p Value (t test)
Cortical gray matter	178 ± 41	227 ± 26	0.001
Deep nuclear gray matter	10.8 ± 4.2	13.8 ± 5.2	0.02
Myelinated white matter	13.5 ± 5.8	20.8 ± 12	0.02
Unmyelinated white matter	202 ± 41	207 ±78	0.9
Total cerebral tissue	406 ± 57	457 ± 67	0.003
Cerebrospinal fluid	46 ± 22	29 ± 17	0.01

Fifty-three children (19%) had severely delayed development (with scores more than 3 SD below the mean), and a further 32 children (11%) had scores between 2 SD and 3 SD below the mean. Ten percent had severe neuromotor disability, 7% were blind or perceived light only, and 3% had hearing loss that was not correctable or required hearing aids. Overall, 49% had disability and 23% were considered to be severely disabled (Wood *et al.*, 2000). Eighteen percent of these children had cerebral palsy: this was of a diplegic pattern in about half (54%), 10% had hemiplegia, and 24% had quadriplegia (Wood *et al.*, 2000). The mean head circumference, a measure of brain growth, was 1.6 SD below the mean for corrected age and significantly lower in those with any severe disability than in those with no disability. However, head size is not discriminatory at this age because 24% children with no disability had a head circumference more than 2 SD below the mean (Wood *et al.*, 2000).

The same group of children (n=241) were seen again aged 6 years with a comparison group of classmates born at full term (Marlow *et al.*, 2005). Cognitive impairment (defined as results more than 2 SD below the mean) was present in 41% of the children born extremely preterm when the results were compared with those for their classmates: the overall cognitive score for the children who had been born extremely preterm was 82.1 ± 19.2 (compared with 105.7 ± 11.8 for the classmate controls). The rate of severe disability (more than 3 SD below the mean) was 22%, moderate disability (more than 2 but not more than 3 SD below the mean) affected 24%, and 34% were mildly disabled (more than 1 but not more than 2 SD below the mean).

Disabling cerebral palsy was present in 12% of the survivors of this very vulnerable group (Marlow *et al.*, 2005). Forty-nine (20%) had spastic or dyskinetic cerebral palsy and a further nine had abnormal neurological signs (hypotonia), whereas none in the

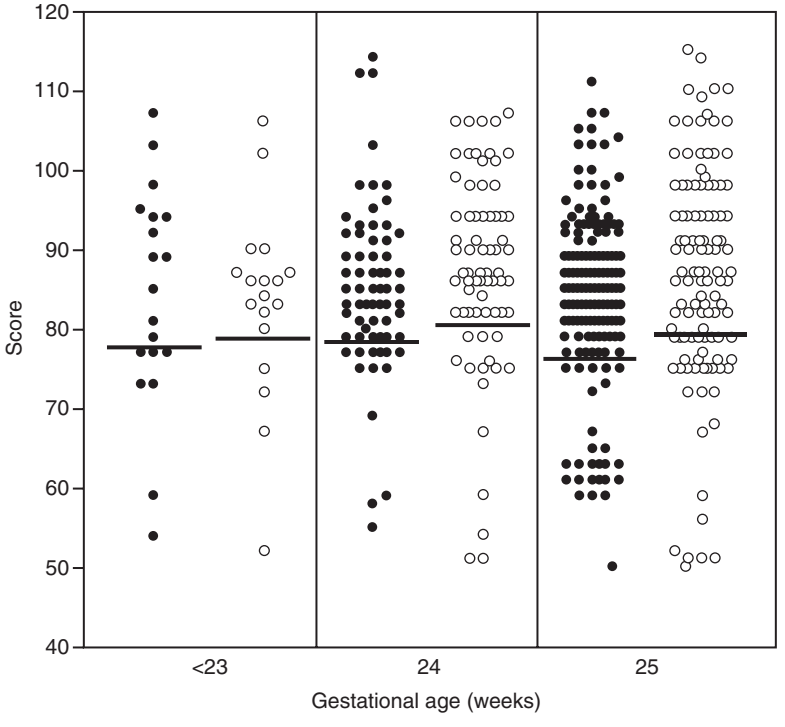

Fig. 19.8 Individual Bayley Scale scores at 30 months according to gestation. Mental Development Index (MDI) (231 infants) scores are indicated by solid circles and Psychomotor Development Index (PDI) (225 infants) scores by open circles. The horizontal lines represent means. (From Wood *et al.*, 2000.)

control group had abnormal neurological conditions. Thirty-five children (15%) had signs of spastic diplegia, four had hemiplegia, nine had quadriplegia, and one child had dyskinetic cerebral palsy. Of these 35 children with cerebral palsy, 15 (6%) had severe motor disability (i.e., were not walking) and another 15 were walking independently but with an abnormal gait (moderate motor disability). The mean ± SD cognitive score for these 30 children was 49 ± 17. The EPICURE study group reported that the remaining 19 children with cerebral palsy had no evidence of clinically important functional difficulties relating to gait or hand use and a mean cognitive score of 81 ± 19. Twelve children with diplegia (34%) had mild motor disability, as compared with only 7% of children with other types of cerebral palsy. Cerebral palsy was more common among boys than girls (26% vs. 14%).

Relatively few children in this group had severe sensory morbidity, an observation remarkably similar to that reported by Hack and colleagues (2002) and Cooke (2004) in young adults (see below). Four children were blind, and two could see only light (severe disability, 2%), and five of these children had received treatment for retinopathy of prematurity (Marlow *et al.*, 2005).

Adolescence

Adolescence is a critical period of brain structural reorganization and maturation of cognitive abilities. The hypothesis that this relatively late developmental reorganization may be altered in individuals who were born preterm was examined by Allin and colleagues (2007), who carried out longitudinal neuropsychological testing in 94 very preterm individuals (born before 33 weeks' gestation) and 44 term-born individuals at mean ages of 15.3 years (adolescence) and 19.5 years (young adulthood, see below). Full-scale IQ testing results, as well as verbal and performance IQ subscales and phonological verbal fluency were significantly lower in the preterm group than the term group at both ages. Semantic verbal fluency increased significantly in the term group between the two periods of measurement, but not in the preterm group (Allin *et al.*, 2007).

Young adulthood

Neonatal intensive care has been practiced for long enough now for some of its graduates to have reached adulthood. Two carefully conducted studies, one in the United States and the other in Europe, looked

at the outcome for young adults who had been of very low birthweight (<1500 g, VLBW). Hack and colleagues in Cleveland, Ohio, compared the outcome of 242 VLBW survivors born between 1977 and 1979 with that of 233 controls (Hack *et al.*, 2002). When aged 20 years, they were assessed for education, cognition, chronic illness, and risk-taking behavior. Compared with people who had been born at term, fewer VLBW young adults had graduated from high school (74% vs. 83%; $p = 0.04$), VLBW men (but not women) were less likely to be studying (30% vs. 53%; $p = 0.002$), and young people born with a VLBW had lower mean IQs (87 vs. 920). People born with VLBW had higher rates of neurosensory impairment (10% vs. <1%) and were shorter than controls (10% had heights below the third percentile, compared with 5% in the control population). Six percent of the people born with VLBW in the late 1970s had cerebral palsy (compared with none in the control group), 2% had hydrocephalus, 2% were blind, and 2% had epilepsy (compared with 0.4% in the control group) (Hack *et al.*, 2002).

Cooke in Liverpool, United Kingdom, considered a similarly aged group of people born with VLBW and compared their outcome by postal questionnaire with that of term classmate controls aged between 10 and 22 years (Cooke, 2004). Academic achievements among those born preterm were less than for those born at term, although as many in each group progressed beyond secondary education. Significantly, more of those born preterm had vocational qualifications (54% vs. 23%) but fewer had a university education (23% vs. 58%). Unemployment rates were low overall, but higher in those born preterm.

In both North America and England, the young people who were born prematurely were as likely as their term-born peers to smoke, but were significantly less likely to undertake other risky forms of behavior, such as drinking alcohol, smoking cannabis, or (in the United Kingdom) taking heroin or the drug Ecstasy (Hack *et al.*, 2002; Cooke, 2004). The UK study looked at other lifestyle issues and concluded that people born significantly preterm were less satisfied with their appearance and more likely to use a regular prescription medicine (Hack *et al.*, 2002; Cooke, 2004).

Executive functioning is impaired in young adults who were born very preterm (<33 weeks of gestation). Nosarti *et al.* (2007) studied 61 subjects and 64 controls at around 22 years of age. Participants, who had been very preterm, showed specific impairments in tasks involving response inhibition and mental flexibility, even when adjusting for IQ, gender, and age. There were no significant associations between executive function test scores and perinatal variables or neonatal ultrasound classification. The results suggested that, although free from major physical disability, very preterm young adults perform worse than controls on tasks involving selective aspects of executive processing, such as mental flexibility and response inhibition.

There was an improvement in outcomes in infants of 22–26 weeks' gestation over three two-year epochs (1993–4, 1995–6, 1997–8) (Vohr *et al.*, 2005) with a significant reduction in neurodevelopmental impairment from 50% to 45% and specific declines in Bayley MDI and PDI scores. However, the decline in neurodevelopmental impairment almost exactly balanced the increase in survival (from 55% to 61%), and consequently the absolute number of children with impairment remained unchanged (Watts & Saigal, 2006). The Dutch POPS study (Leiden Follow-Up Project on Prematurity) was even more pessimistic (Rijken *et al.*, 2003), with 92% of 23–24 weeks' gestation infants and 64% of those born at 25 weeks having adverse outcomes (defined in this study as death or abnormal development). This influenced clinical practice in the Netherlands to the extent that a number of hospitals stopped offering intensive support to babies born below 26 weeks' gestation.

Interventions

Effect of interventions on cognitive function

A newborn baby's physical environment seems to have an effect. In Nottingham, United Kingdom, Mann and colleagues (1986) studied the effect of alternating night and day on sleep, feeding, and weight gain in a randomized controlled trial. Babies from a nursery where the intensity of light and noise was reduced for 12 hours at night spent longer sleeping and less time feeding and gained more weight than those from a control nursery where the intensity of light and noise was not reduced. Differences became apparent only after discharge home and were still detectable three months after the expected date of delivery, when infants from the night and day nursery were an average of 0.5 kg heavier ($p < 0.02$). These findings suggested that physical environment has an effect (either

direct or indirect) on the subsequent behavior of preterm infants and that there was benefit from creating a separate night and day environment (Mann *et al*., 1986).

The environment was also studied by the Newborn Individualized Developmental Care and Assessment Program (NIDCAP). This philosophy of neonatal nursing care involves promoting infant development and parenting and making the neonatal intensive care unit's environment less stressful to babies by reducing the levels of light and noise (Als & Gibes, 1986; Als *et al*., 1994, 1995). The NIDCAP approach was subjected to a small randomized controlled trial in Boston, Massachusetts. Forty-three infants who weighed less than 1250 g at birth were entered over a 21-month period between 1984 and 1986. They were either nursed by a team of specially trained nurses, or given routine care. The results were striking. Six (33%) of the control group developed chronic lung disease, compared with none in the experimental group. Four (22%) control infants developed PVH with parenchymal involvement, compared with none in the experimental group. Babies who received what has come to be known as individualized nursing did much better than controls. On average, they fed by bottle sooner than controls (104 days vs. 59 days), spent less time in hospital (151 days vs. 87 days), and were in oxygen for a shorter period (139 days vs. 57 days). At 42 weeks of postconceptional age, the NIDCAP babies generally did better in terms of their posture, tone, and movement. At 9 months, the experimental group had significantly higher mean Bayley scores than controls (MDI 118 vs. 94 and PDI 101 vs. 84). The paper was accompanied by a rapturous editorial (Merenstein, 1994), and followed by serious criticism in the correspondence columns of the *Journal of the American Medical Association* (Garland, 1995; Lacy, 1995; Ohlsson, 1995; Saigal & Streiner, 1995; Sepkowitz, 1995). The correspondents made a number of points: the study was carried out before artificial surfactant became available, and the rate of severe PVH in the control group was very high. The study was also criticized on the basis that the staff would have been aware which group the infants were in, the relatively small sample size, and the wide variability of some of the results (for example, the mean ± SD number of days in oxygen was reported as 139 ± 166).

In 2004, Sizun and Westrup reviewed the evidence base for NIDCAP and set out nicely the theoretical framework underlying developmental care: the evidence that implicates stress as an adverse factor on the very immature developing brain, probably through a corticosteroid effect, provides a biologically plausible mechanism for the benefits claimed by the proponents of individualized developmental care (see also Westrup *et al*., 2000). A Cochrane review observed limited benefits with respect to necrotizing enterocolitis, improved family outcome, and moderate–severe chronic lung disease, but an increase in mild lung disease and in the length of stay by infants receiving developmental care (Symington & Pinelli, 2006). Although the authors concluded that "before a clear direction for practice can be supported, evidence demonstrating more consistent effects of developmental care interventions on important short- and long-term clinical outcomes is needed," individualized care has been enthusiastically and uncritically adopted by neonatal nurses. A recent nurse-delivered, parent-focused interaction program had no measurable effects on short-term infant neurobehavioral function, mother–child interaction, or parenting stresses (Glazebrook *et al*., 2007).

Psychologists describe children being "doubly vulnerable" through both biological and environmental disadvantage (e.g., Guralnick, 1998). Separation of these two issues is difficult when assessing the effects of intervention programs. For example, the Infant Health and Development Program was a US national collaborative study aimed at examining the efficacy of combining early child development and family support services with pediatric follow-up to reduce the incidence of health and developmental problems among low birthweight, preterm infants (Ramey *et al*., 1992). The outcome was affected by whether a child's mother had been to college and only children whose mothers had a high school education or less showed significant enhancement in IQ scores at 3 years. But birthweight had an effect too: the lighter (< 2000 g) low birthweight preterm children of white mothers with some college education were less influenced by the intervention than the corresponding heavier children (Brooks-Gunn *et al*., 1992), suggesting that the intrauterine environment also had some effect.

In 1987, Shonkoff and Hauser-Cram published a metaanalysis of 31 early intervention programs for children up to 3 years old with disability due to an acquired impairment. This showed that participation was associated with an average gain of 0.62 SD for measurements of cognitive development, and that the

most effective programs targeted both parents and children. Guralnick (1998) reviewed the effectiveness of early intervention programs at improving cognitive functioning. He pointed out that children who were vulnerable through disadvantage and those who already had disabilities were likely to have an increasing gap between their level of cognition and those of healthy controls during the first five years. This meant that, if there was no intervention, children considered vulnerable because of their social circumstances ended up with an apparent IQ between 0.5 and 1.5 standard deviations (SDs) lower at 5 years compared with earlier assessment. Children with established disabilities started at a lower level and their IQ at 5 years was approximately between 0.5 and 0.75 SDs lower, i.e., between 8 and 12 IQ points. Guralnick (1998) concluded that there was now "unequivocal evidence" that early intervention can reduce this apparent decline.

Three other randomized studies are worth considering in some detail: the Abecedarian Project (Ramey & Campbell, 1984), the Vermont study (Rauh *et al.*, 1988), and the Infant Health and Development Program (McCormick *et al.*, 1991; McCarton *et al.*, 1997). The type and frequency of intervention and the socioeconomic status of the families were rather different in each.

The Abecedarian Project (Ramey & Campbell, 1984) examined the effect of early educational intervention for children of poor families. Subjects were randomly assigned to one of four interventions: educational treatment from infancy until the age of 8 in public school; preschool treatment only (from infancy until 5 years old); primary school treatment only (age 5–8 years); and an untreated control group. At 4 years of age, only 3% of the group that received an intervention had an IQ below 70 compared with 18% of controls, and their IQ was on average 0.82 SD higher. The advantage was most marked among children whose mother's IQ was below 70 (Martin *et al.*, 1990). The preschool intervention appeared to have a continuing positive effect on intellectual development and academic achievement, with a detectable effect at 12 years. Cognitive and academic achievement increased with longer treatment (Campbell & Ramey, 1994).

The Vermont study was a low-intensity intervention, aimed at improving maternal adjustment to caring for preterm infants of birthweight below 2200 g (Rauh *et al.*, 1988). There were only 11 sessions, which started during the final week of hospitalization and continued for three months at home. Fifty-four low birthweight babies were randomly assigned to experimental or control conditions, and the results were compared with the outcome for 28 full-term, normal birthweight infants. By 36 and 48 months there were significant group differences on the General Cognitive Index of the McCarthy Scales: the low birthweight controls were the most disadvantaged, but the low birthweight experimental group had caught up with the normal birthweight group.

The third North American study was the Infant Health and Development Program, a remarkable multicenter randomized controlled trial, where the intervention was applied from birth to 3 years. Three hundred and seventy-seven infants were assigned to the intervention group and there were 608 controls and stratification of low birthweight premature infants to below and above 2001 g (Ramey *et al.*, 1992). The intervention group received home visits from the time of discharge from the maternity unit until they were 3 years old, as well as special schooling and parent group meetings between 1 and 3 years of age. Cognitive and behavior problem scores were significantly better for the intervention group at 24 and 36 months (Brooks-Gunn *et al.*, 1993). The gains were most pronounced for receptive language and visuomotor and visuospatial skills. By the time the children were 5 years old, there was no difference for the whole group. However, the heavier low birthweight children who had received the intervention had average full-scale IQ scores which were 3.7 points higher ($p = 0.03$) and mean verbal IQ scores which were 4.2 points higher ($p = 0.02$) than those who had been lighter babies (Brooks-Gunn *et al.*, 1994). At 8 years, the children in the lighter low birthweight cohort were still similar to controls, and those in the heavier low birthweight group still showed advantages. The children who had been larger babies had a full-scale IQ that was 4.4 points higher ($p = 0.007$), a better verbal IQ score (4.2 points higher, $p = 0.01$), a higher performance IQ score (3.9 points higher, $p = 0.02$), and better mathematics (4.8 points higher, $p = 0.04$) and receptive vocabulary scores (6.7 points higher, $p = 0.001$) (McCarton *et al.*, 1997). The general observation was that lower birthweight children did less well and this was also found by other studies (e.g., McCormick *et al.*, 1996). An interesting finding related to the effect of environmental stimulation: children whose mothers had attended college did not exhibit significant

enhancement, but those whose mothers had had a high school education or less benefited from the intervention (Brooks-Gunn *et al.*, 1992).

A study in Avon, England, also showed a small advantage for a structured developmental program over social support, particularly for the smallest infants (birthweights <1250 g) and those with brain injuries (Anonymous, 1998). Here, a randomized controlled trial compared Portage, a home developmental education program, with nondirectional counseling by parent advisers. Social variables confounded the results, but, when linear regression analysis was applied, there was a positive effect due to Portage (+4.3 general quotient points, 95% CI 1.6 to 7.0) and the parent adviser group (+3.4 general quotient points, 95% CI 1.4 to 6.1).

In conclusion, it seems that early and sustained supportive interventions may result in improved cognition, but there is a powerful environmental effect. Infants whose growth was restricted in utero seemed to benefit least (Zeskind & Ramey, 1978, 1981). The effects were substantial enough for the UK government to introduce a support program aimed at the children in the most deprived areas, the SureStart program. However, a study of 35 000 children in England between 2001 and 2006 suggested they were no further advanced now than they were before the scheme was introduced. After taking account of deprivation, language, age, and sex, the only significant change over time was a slight decline in picture vocabulary (Merrell *et al.*, 2007).

Effect of early interventions on children with cerebral palsy

There have been a number of papers on physical therapy for children with cerebral palsy, but few randomized controlled trials. Studies carried out between 1966 and 1994 were reviewed by Hur (1995) and Turnbull (1993). There have been three trials where randomization was blind, and only one in which the control group received no intervention at all (Goodman *et al.*, 1985; Piper *et al.*, 1986; Weindling *et al.*, 1996).

In South Africa, Goodman and colleagues (1985) used a neurodevelopmental assessment at 3 months to identify babies who were considered to be likely to benefit from neurodevelopmental therapy. Infants below 34 weeks' gestation and 1700 g were randomly assigned to a hospital-based program with additional home exercises. At 12 months, at-risk infants still had lower developmental quotients than normal infants (suggesting that the neurodevelopmental assessment scale was valid) and there was no difference at 6 years. However, the locomotor score of the at-risk children was significantly below that of normal children, and 6/24 of the at-risk children had cerebral palsy, compared with 0/25 of those who had been considered normal at 3 months (Rothberg *et al.*, 1991).

In Montreal, Piper *et al.* (1986) examined the effect of neurodevelopmental therapy aimed at optimizing position and movements for an "at-risk" population, defined as infants with a birthweight below 1500 g and others who had been exposed to significant hypoxic ischemia. There was no difference in neurodevelopment, although infants weighing below 750 g at birth, regardless of group assignment, showed poorer growth and development than their heavier peers.

In Liverpool, we also examined the hypothesis that infants at high risk of cerebral palsy might benefit from early neurodevelopmental therapy. Infants with abnormal cranial ultrasound scans were randomized at around term to early neurodevelopmental therapy or standard treatment where physical therapy was delayed until abnormal physical signs became apparent. There was no difference in outcome at 12 and 30 months (Weindling *et al.*, 1996).

The studies described in this section illustrate some of the difficulties in devising and assessing therapy programs aimed at improving motor function. The patterns of disability of the children studied are usually heterogeneous. The nature of the intervention varies and there is no clear guidance from the scientific literature as to which intervention is most likely to be effective. There is difficulty in defining a robust criterion-referenced outcome measure. Ideally, account should be taken of confounding factors, such as social and family circumstances, although this should not matter if the intervention is randomized and controlled. However, a family may seek out other interventions without the knowledge of the investigator. Overall, no study aimed at improving the motor development of preterm vulnerable infants has shown an effect (for a review, see Weindling *et al.*, 2007).

Pharmacological interventions

There have been several pharmacological interventions particularly intended to reduce the incidence of PVH. Of these, corticosteroids given to the mother antenatally for its effect on the fetus, and indometacin given to the neonate after birth, have an effect and have found their way into routine clinical practice. Two

other drugs, vitamin E and ethamsylate, have been shown by randomized trial to have some effect in reducing PVH. Vitamin E is an antioxidant and it has been suggested that it protects endothelial cell membranes from oxidative damage and disruption, limiting bleeding, and its spread from the subependyma into the ventricles, but it has not entered general clinical practice (Chiswick *et al.*, 1983; Sinha *et al.*, 1985). Ethamsylate also stabilizes capillary membranes and inhibits prostaglandin synthesis (Morgan *et al.*, 1981; Benson *et al.*, 1986). Phenobarbital either before or after birth (e.g., Shankararn *et al.*, 1997) has not been found to be effective.

The most outstanding success story has been the increasingly widespread use of antenatal steroids. The drug was first used to reduce the incidence of respiratory distress in preterm infants (see overviews by Crowley *et al.*, 1990; Roberts & Dalziel, 2007). This effect is certainly achieved (RR 0.66, 95% CI 0.59 to 0.73). The risk of dying is reduced by 40% (RR 0.69, 95% CI 0.58 to 0.81). It is particularly relevant to note that PVH is also significantly reduced in infants who have been exposed to steroid before delivery (RR 0.54, 95% CI 0.43 to 0.69).

There is convincing evidence from a rat model that corticosteroids have a remarkable brain-protective effect against hypoxia–ischemia (Barks *et al.*, 1991). The effect of neonatal corticosteroid administration on brain damage caused by cerebral hypoxia was investigated by the administration of the various doses of dexamethasone in 7-day-old rats. These animals were subjected to a unilateral cerebral hypoxic–ischemic insult, causing a large unilateral cerebral infarct in 79% of controls, whereas all those which had been given dexamethasone for 3 days before the hypoxic–ischemic insults had no infarction. The neuroprotective effect of pretreatment dexamethasone was dose and time dependent. Treatment with dexamethasone after hypoxia or with lower doses before the insult did not prevent infarction. The neuroprotective effect was only seen when the dexamethasone was given 24 hours before hypoxic ischemia, and not when it was given immediately before hypoxic ischemia. These experiments appeared to confirm that fetal exposure to corticosteroids has a brain-protective effect when there is subsequent (or perhaps ongoing) hypoxic ischemia. They supported earlier work using a 3-day-old rat model, where both dexamethasone and indometacin had similarly sized protective effects (Pappius & Wolfe, 1983).

Although the precise mode of action of steroids remains uncertain, it is likely that they also affect the production of oxygen-derived free radicals. Hydrocortisone seems to be relatively ineffective compared with prednisolone and methylprednisolone (Hall, 1992). The effect seems to be due to the presence of a 1,2 double bond found in methylprednisolone and prednisolone, but not in hydrocortisone. The efficacy of prednisolone and methylprednisolone – and presumably betamethasone, which is administered to women at risk of delivering prematurely – is not related to their antiinflammatory effect (Hall, 1992), but to the ability of the steroid to inhibit central nervous system (CNS) membrane lipid peroxidation (Braughler, 1985). Methylprednisolone is three times as effective as prednisolone (Hall, 1992).

Several authors have reported a reduction in the incidence of PVH in preterm infants exposed to antenatal steroids and also delivered by cesarian section, supporting a view that hypoxic ischemia during the immediate prenatal and intrapartum period is likely to be damaging (e.g., Ment *et al.*, 1995; Roberts & Dalziel, 2007). One possible mechanism is that corticosteroids exercise their effect through maturation of the germinal matrix microvasculature in the preterm infant, since indometacin has been shown to induce basement membrane production in the germinal matrix microvasculature of the newborn beagle pup and it too has a protective effect on PVH (Ment *et al.*, 1992, 1994a, b).

The prostaglandin synthetase inhibitor indometacin, a nonsteroidal antiinflammatory drug, reduces PVH (Fowlie, 1997). The clinical reason for giving indometacin was to reduce the incidence of symptomatic patent ductus arteriosus (PDA), which is common in preterm infants and may adversely affect cerebral perfusion through a "steal." Closure of the PDA was achieved in treated infants but prophylactic indometacin also significantly reduced the incidence of grade 3 and 4 IVH in treated infants (pooled RR 0.60, 95% CI 0.43 to 0.83). There was no evidence that prophylactic indometacin prevented the progression of grade 1 IVH if it was present before starting the drug.

Although indometacin reduces blood flow to organs, particularly the brain (Edwards *et al.*, 1990), kidneys (Cifuentes *et al.*, 1979), and gut (Coombs *et al.*, 1990), there is no evidence to suggest that prophylactic indometacin is associated with long-term adverse effects (Fowlie, 1997). Nor does it seem to benefit development. In randomized controlled trials, Bandstra and colleagues (1988) found no effect on

Bayley MDI and PDI at 12 and 24 months and Ment *et al.* (1994b, 1999) found no difference at 36 months between indometacin and placebo groups in terms of blindness, deafness, general development, or the incidence of cerebral palsy.

During hypoxic ischemia, adenosine is utilized and there is accumulation of purine metabolites. When reperfusion occurs, oxygen is again available and superoxide free radicals are produced during the oxidation of hypoxanthine by xanthine oxidase, which is inhibited by allopurinol. One study where allopurinol was given to preterm babies after birth did not reduce the incidence of PVL or WMI, but gave a useful insight into a causative mechanism of a number of neonatal diseases (Russell & Cooke, 1995), supporting a view that hypoxic ischemia and reperfusion are important causes of cerebral lesions during the perinatal period. Infants between 24 and 32 weeks' gestation were randomly assigned to receive enteral allopurinol or a placebo daily for 7 days after birth. There was no difference in the development of PVL (4.4% in the allopurinol group vs. 2.6% in the controls) or PVH with brain parenchymal involvement (6.8% in the allopurinol group vs. 5.1% in the controls). However, just after birth there were significantly higher plasma hypoxanthine concentrations in infants who subsequently developed PVL, chronic lung disease, and retinopathy of prematurity compared with controls (median hypoxanthine concentrations for PVL and porencephaly were 13.8 μmol/l and 12.7 μmol/l, respectively, vs. 7.0 μmol/l in healthy controls). Thus, hypoxic ischemia and the generation of oxygen-derived free radicals may play a part in the etiology of PVL and porencephaly due to parenchymal involvement by PVH may also be due in part to ischemia–reperfusion injury.

Nutrition

Nutrition after delivery is important (e.g., Cooke, 2006). Infants born significantly prematurely are particularly vulnerable to deficiencies of long-chain polyunsaturated fatty acids because the placenta selectively transports arachidonic acid and docosahexaenoic acid (DHA) only during the third trimester. ω-3 (*n*-3) and ω-6 (*n*-6) essential fatty acids appear to be particularly important (for reviews, see Uauy-Dagach & Mena, 1995; Simmer *et al.*, 2008). ω-3 refers to a double bond in position three of the carbon chain, counting from the omega end of the molecule. The main ω-3 polyunsaturated fatty acids are α-linolenic acid, eicosapentanoic acid, and DHA. The importance of these acids is that human tissue is unable to introduce a double bond before carbon 9. The clinical effects of ω-3 essential fatty acid deficiency are abnormal visual function and a peripheral neuropathy. There are particularly high concentrations of DHA in the cerebral cortex and retina, where DHA makes up about 50% of fatty acids in the phospholipids. Arachidonic acid is formed from the ω-6 fatty acid, linoleic acid. The assessment of vision has been considered a particularly sensitive indicator of the effects of ω-3 fatty acids, and infants fed on diets rich in DHA have been shown to have better visual acuity than those fed on infant formulae without DHA supplementation (Uauy-Dagach & Mena, 1995). Farquharson and colleagues (1995) measured the fatty acid composition of brain cortex phospholipids of infants dying before 6 months because of sudden infant death syndrome and found that the cerebral cortex of infants who had been breastfed contained more DHA than that of infants who had been fed on a cow's milk-based formula. This can be linked to the observation that breastfed infants (with higher plasma and red blood cell DHA concentrations throughout the first year of life) had higher stereo acuity at 3 years (Birch *et al.*, 1993). Breast milk has also been shown to benefit the developmental outcome of extremely low birthweight infants assessed at 18 months of age. For every 10 ml/kg per day increase in breast milk ingestion, Vohr and colleagues (2006) showed that the Bayley MDI increased by 0.53 points, the PDI increased by 0.63 points, a Behavior Rating Scale percentile score increased by 0.82 points, and the likelihood of rehospitalization decreased by 6%. Add to this the observation that when low birthweight preterm infants fed human milk by gastric tube were compared with those fed milk formula by the same route, there was a mean 8.3 IQ advantage when they were 8 years old (Lucas *et al.*, 1992), and the advantage conferred by ω-3 fatty acids seems clear.

However, there is a potential disadvantage. Oxygen-derived free radicals damage lipid membranes by breaking the double bonds in the carbon chains of the fatty acids (Uauy-Dagach & Mena, 1995). Membranes, which are rich in arachidonic acid, eicosapentanoic acid, and DHA, are therefore particularly susceptible to oxidative damage and infants born very prematurely are deficient in antioxidants. Comparing the antioxidant status of full-term babies born by normal delivery with a preterm group (mean gestation 34 weeks), there was greater activity of catalase (2.10 ± 0.16 mBU/mg

protein vs. 1.86 ± 0.16 mBU/mg protein), glutathione peroxidase activity (1.25 ± 0.07 mU/mg protein vs. 0.79 ± 0.19 mU/mg protein) and Cu/Zn-superoxide dismutase (Georgeson *et al.*, 2002).

The endocrine milieu

Babies born at less than 26 weeks' gestation have a very different endocrine milieu than those born at term. The two endocrine systems that seem to be particularly important are the hypothalamic–pituitary–adrenal (HPA) and the hypothalamic–pituitary–thyroid (HPT) axes. It is likely that there is interaction between the two, but the evidence for their possible effects will first be considered separately.

Hypothalamic–pituitary–adrenal axis

Steroids have a powerful effect on brain development. Vohr and colleagues (2005) found that antenatal steroid administration was the only study intervention between 1993 and 1998 associated with improved outcomes at 18–22 months' corrected age. There have, however, been serious concerns about long-term side-effects (neurodevelopmental impairment and impaired growth) (Barrington, 2001). These are supported by observations that administration of corticosteroids has serious adverse effects on the growth of brains in animals. For example, a mouse model has shown that repeated doses of antenatal corticosteroids were associated with a decrease in brain surface area and in the whole cortex convolution index, a measure of cortical surface complexity (Modi *et al.*, 2001). Most significantly, the developing human brain also seems to be vulnerable (Fig. 19.9). Several studies have shown an increased rate of neurodevelopmental disability associated with corticosteroid used both before (Halliday, 2004; Yeh *et al.*, 2004) and after (Barrington, 2001; Halliday, 2004) birth.

Sizonenko *et al.* (2006) reviewed the likely mechanisms. Glucocorticoids are essential for the regulation of neurogenesis and neuronal survival, and glucocorticoid administration produced altered numbers of neural cells and an alteration in synaptic function in the forebrain, expressed more in the male brain than that of the female (Kreider *et al.*, 2006).

These serious concerns about side-effects have led to a reduction in the clinical use of postnatal steroids (Shinwell *et al.*, 2007).

Hypothalamic-pituitary-thyroid axis

Thyroid hormones are essential for normal brain development (Morreale de Escobar *et al.*, 2004; Williams *et al.*, 2007). The neurological deficits of congenital hypothyroidism are well characterized and include IQ, visuomotor, and visuospatial deficits as well as problems with reading, comprehension, memory, attention, and arithmetical skills (Salerno *et al.*, 1999; Rovet & Hepworth, 2001; Song *et al.*, 2001). In animal models, thyroxine deficiency during early neurogenesis causes abnormalities of specific structures such as the hippocampus, temporal and sensorimotor cortices (Iniguez *et al.*, 1996; Cai *et al.*, 2000; Lavado-Autric *et al.*, 2003; Auso *et al.*, 2004; Bernal, 2007). Perinatal hypothyroidism in rats altered the orientation of cortical layers and the density and size of neurons (Eayrs, 1953; Eayrs & Horn, 1955). Thyroid hormones also increase proliferation

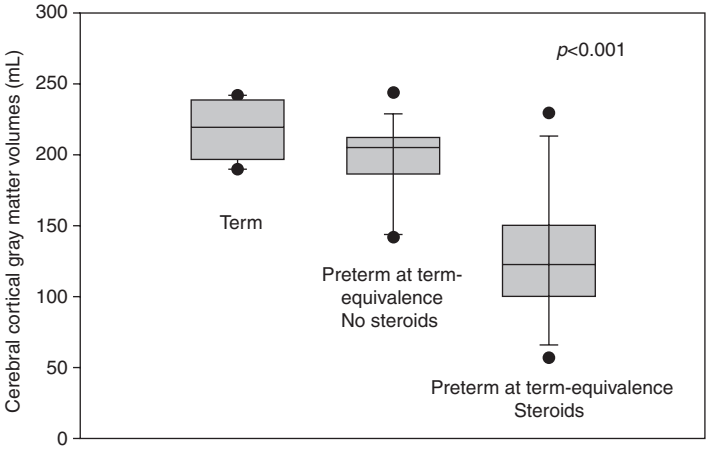

Fig. 19.9 Cerebral cortical grey matter volumes measured by volumetric magnetic resonance imaging in healthy term babies, compared with preterm babies at term-equivalence who did not receive postnatal steroids, and those who received postnatal corticosteroids. The reduction in cortical gray matter volume seen in preterm infants who received postnatal corticosteroids was significant. (Redrawn from Murphy *et al.*, 2001.)

Table 19.3 Neurodevelopmental outcome and low blood thyroxine concentrations in infants below 32 weeks or of birth weight <1500 g (Ng, 2008)

Study	Participants	Outcome
Reuss *et al.* (1996)	463 infants born below 33 weeks' gestation	Fourfold increased risk of disabling cerebral palsy at 2 years of age associated with hypothyroxinamia group.
Den Ouden *et al.* (1996)	563 infants born below 32 weeks' gestation	Higher incidence of neurological dysfunction at 5 years and 9 years of age associated with hypothyroxinemia group after adjustments for other perinatal factors
Leviton *et al.* (1999)	1414 infants weighing 500–1500 g	Double the risk of cerebral white matter changes associated with hypothyroxinemia
Paul *et al.* (1998)	343 infants weighing less than 1500 grams	Increased mortality and increased incidence of intraventricular hemorrhage associated with hypothyroxinemia group
Lucas *et al.* (1996)	279 infants born below 36 weeks' gestation	Reduction in IQ at 8 years of age associated with low T_3 after adjustment for potential confounding factors
Meijer *et al.* (1992)	563 infants born below 32 weeks' gestation and/or birthweight less than 1500 g	Negative score on the three milestones of development at 2 years of age associated with hypothyroxinemia group

of cerebellar granule cells and affect apoptosis (Nicholson & Altman, 1972; Xiao & Nikodem, 1998).

Evidence is emerging that transient hypothyroxinemia of prematurity (characterized by low blood thyroxine [T_4] concentrations with normal or mildly elevated plasma thyroid-stimulating hormone [TSH] concentrations) may adversely affect extremely premature infants, in whom the HPT system is still developing (LaFranchi, 1999; Williams *et al.*, 2004; La Gamma *et al.*, 2006) (Table 19.3). The fetal thyroid gland does not become functional until 12 weeks' gestation and, during the first trimester, the fetus depends completely on transplacental transfer of maternal T_4 (Calvo *et al.*, 1990; Morreale de Escobar *et al.*, 2004). Fetal thyroid secretion begins at mid-gestation (Lavado-Autric *et al.*, 2003; Bernal, 2007) but significant maternal transfer of T_4 continues until term and the likelihood is that this continues to promote neurodevelopment (Morreale de Escobar *et al.*, 2000, 2004; Obregon *et al.*, 2007). In the second and third trimesters, maternal T_4 continues to be important as T_3 is generated from T_4 in the cerebral cortex, where it is partly bound to specific nuclear receptor isoforms (Morreale de Escobar *et al.*, 2004).

There is uncertainty about what is an appropriate extrauterine blood T_4 concentration (Fisher & Klein, 1981; Chowdhry *et al.*, 1984; van Wassenaer *et al.*, 1997a; Biswas *et al.*, 2002; Williams *et al.*, 2004). A recent large collaborative Scottish study showed that the postnatal surge of T_4 levels, described in term infants, was less marked in babies of 31–34 weeks, absent in infants of 28–30 weeks, and reversed in infants of 23–27 weeks (Williams *et al.*, 2004).

Thyroid supplementation in premature infants

Although an overview of thyroid hormone supplementation for premature infants showed no difference in outcome (Osborn & Hunt, 2007), a study by van Wassenaer and colleagues (1997b, 2005) of T_4 supplementation for infants under 30 weeks showed that, while there was no benefit for the whole group, treated babies below 27 weeks' gestation had improved Bayley MDI and PDI scores at 2 and 10 years. In contrast, the outcome for babies supplemented with T_4 who were born at 29 weeks' gestation and above had a worse outcome than controls. However, this post hoc analysis, which was not prespecified, comprised just 19 babies in the treatment and 27 in the placebo arms. We speculated that the more mature babies may have failed to benefit from thyroxine supplementation because of increased maturation of both the HPT and HPA axes and are currently undertaking an explanatory randomized controlled trial to test this hypothesis.

Conclusions

This chapter has identified the period before 32 weeks as being one where brain development is vulnerable. There are two common brain-damaging conditions that affect preterm infants, PVH and WMI. Although the same population of infants is vulnerable to both conditions, PVH and WMI have different etiologies. The vulnerability of the very premature infant to PVH is due to the characteristics of the cerebral circulation at this period of development. The causes of WMI are

complex and multifactorial and probably relate to the fact that synaptogenesis is occurring at a time when the infant is exposed to various insults: high levels of oxygen (compared to the intrauterine situation) when antioxidants are low, cytokines, hypoxic ischemia, immature endocrine systems, and poor nutrition.

The most striking effect on reducing brain damage in premature infants has been through corticosteroids administered antenatally to the mother. However, when administered in high dose postnatally, they have caused neurodevelopmental impairment.

Programs to improve cognitive function have been more effective than those aimed at motor function. There are, however, considerable methodological difficulties in assessing the effectiveness of such programs; these are related to the heterogeneity of the populations studied and the relatively poor discriminatory power of the tests used.

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Section 5
Chapter
20

Follow-up

Injury and recovery in the developing brain

Koray Özduman and Laura R. Ment

Introduction

Preterm delivery, especially when associated with very low birthweight (VLBW, <1500 g) or extremely low birthweight (ELBW, <1000 g), represents a major insult to the developing brain. VLBW premature infants make up 1%–2% of all live-births, and more than 85% survive the neonatal period (Mc'Intyre *et al.*, 1999). Unfortunately, the increase in survival among premature infants in the 1990s was accompanied by a reported increase in disability (Wilson-Costello *et al.*, 1998). In addition, the brains of preterm children have been shown to be 6% smaller than term controls at school age, and numerous structural and microstructural di erences in brain development have been documented in the preterm population (Reiss *et al.*, 2004).

In contrast, recent reports document improvement in testing scores and academic performance across time for the prematurely born, and imaging studies suggest the development of alternative strategies for language processing in preterm subjects at adolescence. Furthermore, numerous preclinical studies have documented the developing brain's ability to engage in regenerative and recovery mechanisms (Vaccarino & Ment, 2004). Taken together, these findings suggest that preterm birth alters the genetically determined pattern of corticogenesis, and that compensatory mechanisms may promote neural and functional plasticity in the developing brain.

Injury to the developing brain

The most common forms of injury to the developing preterm brain are intraventricular hemorrhage (IVH), cystic periventricular leukomalacia (cPVL), and white matter injury. Other, relatively less common, findings predictive of developmental sequelae include cerebellar hemorrhages and basal ganglia/thalamic lesions (Limperopoulos *et al.*, 2005a,b; Dyet *et al.*, 2006).

Intraventricular hemorrhage

IVH occurs in infants born before 32–34 weeks of gestation and occurs in 20%–25% of VLBW preterm neonates. The incidence of IVH correlates negatively with gestational age (Volpe, 2001a). IVH is most commonly encountered within the first 24 hours after birth, and hemorrhages can progress over 48 hours or more (de Vries *et al.*, 2001). By the end of the first postnatal week 90% of the hemorrhages can be detected at their full extent, and this risk period for IVH is independent of gestational age.

The pathogenesis of IVH is multifactorial. The germinal matrix is the source of both neuronal and glial precursors, and IVH is thought to be secondary to fluctuations in blood flow to the immature microvessels there (Volpe, 2001b). The thin walls of the germinal matrix capillaries, the proximity of the germinal matrix to the ventricular wall, and the absence of a subependymal glial barrier allow pressure to be transmitted during periods of increased flow, and in 80% of the cases the hemorrhage within the germinal matrix ruptures through the ependymal lining into the lateral cerebral ventricle.

Hemorrhages have been traditionally graded using the following nomenclature: grade 1 IVH includes germinal matrix hemorrhage only; grade 2 is characterized by blood within but not distending the ventricular system; and grade 3 hemorrhages are those that fill and acutely distend the ventricular system (Papile *et al.*, 1978). In 3%–15% of infants with IVH, mostly in those who have a moderate to large ventricular hemorrhage, an intraparenchymal hemorrhagic component is also noted (Larroque *et al.*, 2003). Although labeled "grade 4 IVH," some observers believe that this

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is not an extension of the initial hemorrhage but rather periventricular venous infarction (PVI) secondary to obstruction of the terminal vein outflow by a large ipsilateral IVH (Counsell *et al.*, 1999; de Vries *et al.*, 2001).

The neurodevelopmental outcome of IVH, as shown in Table 20.1, depends upon assessment at school age and beyond. Numerous reports have demonstrated that grade 3–4 IVH is a major predictor of adverse outcome (Vohr *et al.*, 2003; Hack *et al.*, 2005), and emerging data suggest lower testing scores for prematurely born children with low-grade hemorrhages when compared with group-matched infants with normal cranial ultrasounds (Patra *et al.*, 2006). Larroque and colleagues (2003) reported that the incidence of cerebral palsy was directly related to grade of IVH in 2364 preterm infants of 22–32 weeks' gestational age at 2 years of age and Sherlock and colleagues (2005) assessed 270 ELBW or very preterm infants at 8 years of age and found that cognitive disability also increased with severity of IVH.

Furthermore, Vollmer and colleagues (2006) noted that those children with left hemisphere grade 4 IVH had greater cognitive consequences than those with right-sided injury. Finally, in the cohort of infants born at 600–1250 g enrolled in the Multicenter Indomethacin IVH Prevention Trials, mortality, cerebral palsy, and mental retardation were all more common in children with grades 3–4 IVH (Ment *et al.*, 2005). More than 50% of this group were deceased, 40% had PVL, and 50% required a ventriculoperitoneal shunt. At age 12 years, almost two-thirds of the children with grade 3–4 IVH had cerebral palsy, 70% had mental retardation, and over 90% required special education services.

Posthemorrhagic hydrocephalus

Posthemorrhagic hydrocephalus is a well-described complication of IVH and is more common in patients with more extensive hemorrhages. It is most commonly a communicating hydrocephalus secondary to obstructive arachnoiditis at the pacchionian granulations, however noncommunicating hydrocephalus secondary to aqueductal obstruction or subependymal scarring may also be observed. In the majority of the cases the hydrocephalus is accompanied by increased head circumference and clinical evidence of raised intracranial pressure. However, due to increased compliance of the neonatal unmyelinated white matter and large subarachnoid spaces in the preterm brain, the signs and symptoms of hydrocephalus may be delayed for several weeks following hemorrhage (Volpe, 1997). Although patients with posthemorrhagic hydrocephalus have been previously reported to experience neurodevelopmental handicap rates of 73% or greater, more recent studies suggest that handicap following posthemorrhagic hydrocephalus is secondary to both the extent of hemorrhage and the treatment modality employed (Whitelaw, 2001; Roland & Hill, 2003).

White matter injury in the developing preterm brain

Long suspected to result in adverse neurodevelopmental outcome, the definition of white matter injury in the preterm brain has changed with the advent of sophisticated imaging technologies. The classical neuropathological description of PVL by Banker and Larroche in 1962 was that of multifocal cysts formed by necrosis within the deep periventricular cerebral white matter. During the 1980s and 1990s, the ultrasonographic demonstration of these cystic lesions correlated well with the development of spastic cerebral palsy in VLBW preterm infants. In addition, a second diagnostic group was described; these children had mild motor impairment, cognitive and behavioral complications, and their symptoms were thought to result from more di use white matter injury (Leviton & Gilles, 1996; Counsell *et al.*, 2003a; Volpe, 2003). Older studies addressing the incidence of PVL are based on the ultrasonographical demonstration of periventricular cysts and report that PVL occurs in 5.7%–16% of all VLBW preterm neonates. Similar to IVH, the incidence of PVL increases with decreasing birthweight and gestational age (Perlman & Rollins, 2000). In contrast, the widespread use of magnetic resonance imaging (MRI) for neonatal imaging has revealed a high incidence of di use white matter abnormalities in the absence of periventricular necrotic foci.

Di use excessive high-signal intensity (DEHSI) is a common finding in premature infants and the incidence increases with advancing postnatal age. The Hammersmith group reported an increase in incidence from 21% in the first postnatal week to 79% at term equivalent (Maalouf *et al.*, 2001). The same group confirmed this high incidence of DEHSI at term equivalent (80%) in a much larger cohort of 119 premature infants (Dyet *et al.*, 2006). Infants with DEHSI have been shown to have both low-pressure ventriculomegaly and increased apparent di usion coe cients in the

Table 20.1 Examples of evidence for injury to developing brain

Modality	Cohort – injury	Outcome
Cognitive/behavioral		
Ancel *et al.* (2006)	N = 2364 PT GA – 22–32 weeks US – Grade 3 IVH, WMD Age 2 years	Percent with CP: 4% with normal US 6% with grade 1–2 IVH 14% with VM 18% with grade 3 IVH 24% with WMD 35% with unilateral cPVL 67% with grade 4 IVH 74% with bilateral cPVL p <0.001
Sherlock *et al.* (2005)	N = 270; BW <1000 g or GA <28 weeks US – IVH; age 8 years	6/6 infants with grade 4 IVH had CP Children with grade 4 IVH scored significantly lower than all others for FSIQ (p <0.001)
Vollmer *et al.* (2006)	N = 668; <33 weeks GA US – grade 4 IVH, cPVL, parenchymal echodensity Age 8 years	Left-sided: FSIQ 80 ± 15 Right-sided: FSIQ 100 ± 19 Bilateral: 86 ± 22 p <0.001
MRI		
Inder *et al.* (1999)	20 PT infants; 14 term control infants Early US diagnosis – PVL MRI term equivalent	PT with PVL had significantly less cortical gray matter at term compared with either PT w/o PVL or term infants (PVL, 157.5 ± 41.5 ml; no PVL, 211.7 ± 25.4 ml; term control 218.8 ± 21.3, p <0.01)
Woodward *et al.* (2006)	167 PT (GA <30 weeks) MRI at term	Moderate to severe WMD: present in 21%; predictive of cognitive delay (OR 3.6; 95% CI 1.5 to 8.7); motor delay (OR 10.3; 95% CI 3.5 to 30.8), CP (OR 9.6; 95% CI 3.2 to 28.3)
Dyet *et al.* (2006)	119 PT infants Serial scans 23–30 weeks' PMA; Griffiths scales at 18 months – CA	At term, 53% infants w/o IVH had VM; 80% had DEHSI; significant relationship was found birth DEHSI and overall DQ (no DEHSI: 111 ± 20; DEHSI: 94 ± 12; severe DEHSI: 92 ± 7.5; p = 0.027)
Volumetric MRI		
Vasileiadis *et al.* (2004)	PT infants at term 12 with IVH; 11 w/o IVH	Cortical gray decreased by 16% in IVH group (p = 0.006)
Kapellou *et al.* (2006)	113 PT neonates serial scans 23–48 weeks' PMA	Surface area to volume scaling exponent significantly related to GA (p <0.001)
Peterson *et al.* (2000)	26 PT, 39 term controls Age 8 years	Volumes for PT significantly less than terms for cortex, basal ganglia, amygdale and hippocampus, and corpus callosum (p <0.001 for all)
Reiss *et al.* (2004)	65 PT subjects 31 term controls Age 8 years	PT males had significantly reduced WM compared with term males (p = 0.021); no difference in WM birth PT and term females
Diffusion tensor MRI		
Huppi *et al.* (2001)	10 PT infants with WMD 10 PT w/o WMD DTI at term equivalent	RA, measure of directionality of diffusion and thus dependent on development of axonal fibers and oligodendroglia, was 20% lower in both the internal capsule and central WM in infants with WMD compared to those w/o WMD (p <0.05)
Krishnan *et al.* (2007)	38 PT infants DTI at term equivalent Griffiths scales – 2 years	Significant negative correlation between mean apparent diffusion coefficient and DQ (p = 0.014)
Functional MRI		
Peterson *et al.* (2002)	26 PT subjects 13 term controls Age 8 years Passive listening task	Preterm semantic activation pattern was similar to term phonologic activation pattern

BW, birthweight; CA, chronological age; CI, confidence interval; CP, cerebral palsy; cPVL, cystic periventricular leukomalacia; DEHSI, diffuse excessive high-signal intensity; DTI, diffusion tensor imaging; FSIQ, Full Scale Intelligence Quotient; GA, gestational age; IVH, intraventricular hemorrhage; MRI, magnetic resonance imaging; N, number; OR, odds ratio; PMA, postmenstrual age; PVL, periventricular leukomalacia; PT, preterm; RA, relative anisotropy; US, ultrasound; VM, ventriculomegaly; WM, white matter; WMD, white matter disease.

central white matter similar to neonates with overt white matter abnormalities, suggesting that DEHSI may represent one end of the spectrum of white matter injury to the developing brain (Counsell *et al.*, 2003b, 2006).

As indicated above the neuropathological findings consist of two distinct injuries. The cystic component is localized deep within the cerebral white matter and characterized by pale areas with cysts (Gilles & Murphy, 1969). Microscopically, these areas are characterized by coagulation necrosis of all cellular elements, with loss of cytoarchitecture and tissue vacuolation (Deguchi *et al.*, 1997). The di use injury is characterized macroscopically by decreased white matter mass, decreased/delayed myelination, thinning of the corpus callosum, and ventriculomegaly (Volpe, 2001c). As studied by conventional pathology the diffuse injury is characterized by di use astrocytosis in the central cerebral white matter, and immunohistochemistry demonstrates preferential death of oligodendrocyte precursors and activated microglia as hallmarks of disease (Haynes *et al.*, 2003). Finally, the pathogenesis of premature cerebral white matter injury is multifactorial and includes ischemia, the inflammatory cascade, and vulnerability of the developing oligodendroglia. Oligodendroglial precursors are particularly susceptible to oxidative damage mediated by reactive oxygen and reactive nitrogen radicals and depletion of glutathione (Inder *et al.*, 2002; Jensen, 2005; Back *et al.*, 2007).

Although the motor consequences of cystic PVL correlate well with periventricular necrotic foci and the extent of ultrasound-demonstrated white matter echolucencies predict cognitive impairment in the prematurely born, the cognitive and behavioral deficits observed in preterm neonates with di use white matter injury do not yet have a clearly defined morphological substrate (Holling & Leviton, 1999). Thus, it is not known whether pure white matter damage, aberrations in axonal guidance or an as-yet undescribed alteration in corticogenesis is responsible for the cognitive impairment found in preterm infants with this injury to developing brain (Haynes *et al.*, 2003; Weiss *et al.*, 2004). Mewes and colleagues (2006) showed that even preterm infants without ultrasound evidence brain injury had moderately decreased white matter volumes at term equivalent. Inder and colleagues (1999) documented loss of cortical gray matter in premature infants with PVL compared with preterms without PVL or term infants, and Marin-Padilla (1997) found cortical dysplasia overlying regions of white matter injury in the prematurely born. As shown in Table 20.1, Woodward and colleagues (2006) examined 167 very preterm infants and showed that moderate to severe white matter abnormalities on MRI at term equivalent predicted the following adverse outcomes at age 2 years: cognitive delay (odds ratio, 3.6: 95% CI 1.5 to 8.7), cerebral palsy (odds ratio 9.6; 95% CI 3.2 to 28.3), and vision and/or hearing impairment (odds ratio 4.2; 95% CI 1.6 to 11.6). Finally, Dyet *et al.* (2006) reported a significant relationship between the presence of DEHSI on term-equivalent MRI and overall developmental quotients for VLBW preterm neonates at 18 months corrected age (N = 57; no DEHSI: 111 ± 20; DEHSI: 94 ± 11.6; severe DEHSI: 92 ± 7.5; $p = 0.027$).

MRI structural and microstructural evidence for injury to preterm brain

Preterm birth is associated with region- (topographical di erences) and compartment- (gray vs. deep gray vs. white matter) specific long-term reductions in brain volume, and these morphological abnormalities are reported to be associated with poorer cognitive outcome (Peterson *et al.*, 2000; Nosarti *et al.*, 2002; Allin *et al.*, 2004; Reiss *et al.*, 2004). Depending on the cohort studied and the age at MRI studies, preterm infants have been shown to have decreased total brain volumes, as well as decreased cortical gray, cortical white, cerebellar, and deep gray volumes and scaling quotients when compared with matched term controls (Vasileiadis *et al.*, 2004; Inder *et al.*, 2005; Kapellou *et al.*, 2006).

MRI studies of older prematurely born subjects suggest persistence of volumetric abnormalities through school age (Table 20.1). A comparison MRI study of 65 prematurely born children and 31 healthy term controls at 8 years of age showed that brains of preterm children were 6% smaller than those of matched controls in all region (Peterson *et al.*, 2000). Significantly reduced volumes were detected in cortical gray, subcortical gray and cerebral white matter, and the white matter loss was most marked in the male infants (Reiss *et al.*, 2004; Gimenez *et al.*, 2006a). A topographical analysis revealed significantly smaller volumes in the preterm children, most prominently in sensorimotor regions but also in the orbitofrontal and temporal lobes, corpus callosum, and hippocampal regions (Rushe *et al.*, 2001; Allin *et al.*, 2004; Gimenez *et al.*, 2006b).

Di usion tensor imaging (DTI) permits the investigation of microstructural alterations in corticogenesis and basic white matter structure in the developing brain (Counsell *et al.*, 2006; Dubois *et al.*, 2006). Fractional anisotropy (FA) describes the degree to which water di usion is restricted in one direction relative to all others and has been shown by several authors to increase with increasing gestational age in the prematurely born (Neil, 2002).

DTI strategies have demonstrated decreased FA in the white matter of preterm infants, even when preterm infants with normal cranial ultrasounds were compared with matched term control infants at term equivalent (Huppi *et al.*, 2001; Counsell *et al.*, 2006). Furthermore, FA has been shown to correlate with outcome in the prematurely born (Krishnan *et al.*, 2007), and Vangberg and colleagues (2006) found decreased FA values in several white matter regions including the corpus callosum, internal capsule, and superior longitudinal fasciculus in preterm subjects compared with term controls at age 15 years. Regions of change also included intra- and interhemispheric association fibers subserving language skills, and Skranes and colleagues (2007) correlated FA values with a wide range of cognitive and behavioral skills in the preterm group.

Evidence for recovery from injury in the developing brain

Several authors have suggested recovery from those neurological insults associated with preterm birth (Table 20.2). Hack and colleagues (2005) found that neurobehavioral testing scores in the first several years of life are poorly predictive of cognitive performance at school age. Goodman and Yude (1996) reported that academic functioning significantly improved between 8 and 14 years in VLBW preterm subjects, and Roth and colleagues (2001) noted that two-thirds of prematurely born children required no special assistance in school at ages 14–15 years. Stewart and colleagues (1999) were unable to detect verbal or cognitive di erences between preterm subjects and term controls at ages 17–18 years. Furthermore, spatial memory, working memory, and recognition memory are all critical to academic success, and Curtis found deficits in these functions in a group of preterm children at 8 years of age compared with matched control subjects; in a follow-up study of the cohort, however, at ages 14–16 years fewer and less pronounced deficits were observed in the preterm subjects compared to the control children, suggesting some improvement of memory in the prematurely born (Curtis *et al.*, 2006).

Studies of VLBW prematurely born young adults also suggest recovery of injury attributable to perinatal events. Hack *et al.* (2002) found that almost three-quarters of an ELBW premature cohort had graduated from high school and 40% were enrolled in college programs, and Saigal *et al.* (2006) found no significant di erences in education or employment variables in 166 ELBW subjects and 145 matched controls at ages 22–25 years.

Our own study of 296 infants born weighing 600–1250 g, who were serially evaluated at 3, 4.5, 6, and 8 years of age, demonstrated that the median Peabody Picture Vocabulary Test-Revised verbal intelligence score (PPVT-R) increased from 88 at 36 months' chronological age (CA) to 99 at 96 months' CA (Ment *et al.*, 2003). When data from the 36 and 96 month CA scores were compared, 45% of children gained 10 points or more, and 12.5% showed a 5- to 9-point improvement. Similar findings were noted for both full-scale and verbal IQ scores, and the study is ongoing. A preliminary analysis of the 90 children for whom scores are now also available at 12 and 16 years found a continued increase in median PPVT-R scores to 102 (Fig. 20.1*a*). Matched term control subjects were added when the preterm subjects were aged 8 years, and the median scores for the control group at ages 8, 12, and 16 years were 102 at each time point. Memory also improved at a rate significantly greater in the preterm subjects when compared with the controls between ages 8 and 16 years (Fig. 20.1*b*).

Structural analyses suggest plasticity of the developing preterm brain

Several authors have commented on the regional vulnerability of the preterm brain. The temporal and frontal regions are responsible for language systems, and preterm subjects ranging from school age to young adulthood have been demonstrated to have changes in the frontotemporal regions bilaterally (Peterson *et al.*, 2000; Gimenez *et al.*, 2006b). In addition, temporal gyrification indices are correspondingly increased in the preterm group, suggesting selective vulnerability in the temporal lobes during the third trimester of gestation (Kesler *et al.*, 2006). In contrast, as shown in Table 20.2, Mewes and colleagues (2006) found accentuated growth of the frontal lobes in PT

Table 20.2 Examples of recovery from injury in the preterm brain

Modality	Cohort – injury	Adaptive mechanism
Cognitive/behavioral		
Ment *et al.* (2003)	296 VLBW subjects Serial evaluations at 3, 4.5, 6, and 8 years PPVT-R Verbal IQ	Median PPVT-R score increased from 88 at 3 years to 99 at 8 years (p <0.001); 45% of children gained 10 points or more; similar findings for FSIQ and VIQ
Hack *et al.* (2005)	200 ELBW subjects Tested at 20 months CA and 8 years	Rate of cognitive impairment (MDC or KABC <70) decreased from 29% at 20 months to 7% at 8 years in children without neurosensory impairments
Saigal *et al.* (2006)	149 ELBW and 133 term subjects Age 22–25 years	No differences in high school graduation, employment/school status or percent who live independently (p >0.1 for all)
Volumetric MRI		
Mewes *et al.* (2006)	23 PT infants scanned at both 32 and 42 weeks' PMA 15 term infants – 42 weeks' PMA	At 42 weeks – gray matter volumes did not differ between PT and term controls; both myelinated and unmyelinated white matter differ (p <0.05 for all regions); significant increase in gray matter growth birth 32–42 weeks in PT group
Fearon *et al.* (2004)	33 PT subjects (<1500 g BW); 18 term siblings Mean age: 23 years	No significant differences in whole brain, gray matter, or hippocampal volumes
Functional MRI		
Santhouse *et al.* (2002)	7 PT subjects with thin CC 9 PT with normal CC 7 term controls Age 18–20 years Auditory phonologic paradigm	PT thin CC group showed greater activation of the right superior temporal gyrus than did both groups of control subjects
Rushe *et al.* (2004)	6 PT with thin CC 6 term controls Age 14 years Phonologic processing	PT showed increased activation in right precentral gyrus, right STG, and right SFG
Ment *et al.* (2006)	14 PT – N neonatal US 10 term controls All subjects with N MRI Age 12 years Auditory phonology task	No difference in out-of-magnet tests for phonology PT activated left STG/MTG + R aMTG Terms deactivated left MTG/STG + R aMTG
Gimenez *et al.* (2005)	14 PT subjects 14 term controls Ages 12–18 years Declarative memory task	PT group had significantly increased BOLD signal in right hippocampus compared with term controls

BOLD, blood oxygen level dependent; CC, corpus callosum; ELBW, extremely low birthweight; PMA, postmenstrual age; PT, preterm; KABC, Kaufman Assessment Battery for Children; MDC, Mental Processing Composite; FSIQ, Full Scale Intelligence Quotient; VIQ, Verbal Intelligence Quotient; PPVT-R, Peabody Picture Vocabulary Test-Revised.

neonates at term, and Kesler and colleagues (2004) reported disproportionately enlarged frontal gray in PT subjects compared with term controls at age 8 years suggesting the development of "hyperfrontality" in the prematurely born. Furthermore, Nosarti (2002) found no di erence in cortical white matter volumes in preterm children assessed at age 15 years when they were compared with matched term control subjects, and Allin and colleagues (2004) studied 33 young adults (mean age 23 years) born preterm and 18 term controls and detected no significant di erences in whole brain, cortical gray, cortical white, or total cerebrospinal fluid (CSF) volumes.

Taken together, although there are no published data describing serial volumetric imaging on prematurely born subjects, these data suggest developmental plasticity of the preterm brain. For preterm subjects, volumetric measurements correlate weakly with cognitive skills. Several investigators have reported the correlation between whole brain, gray matter volumes,

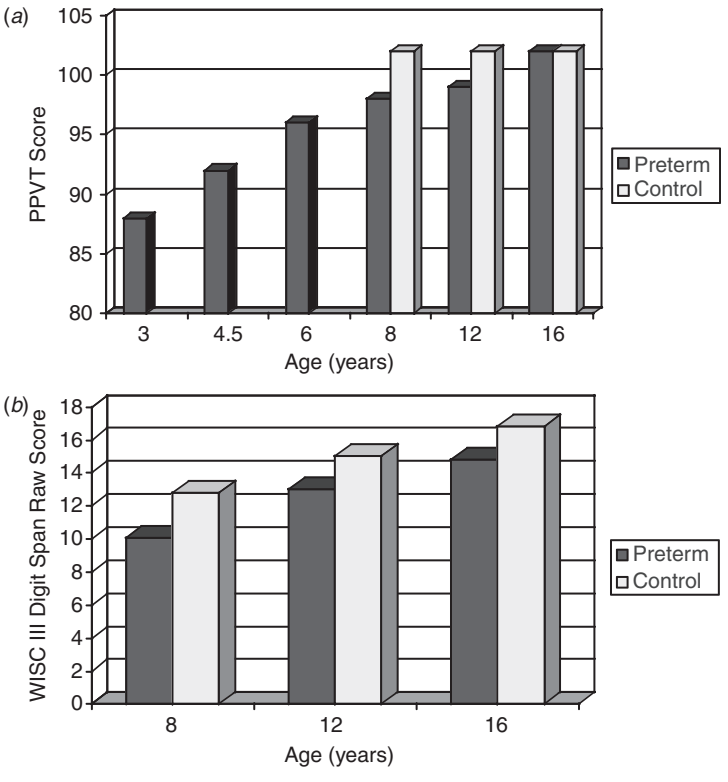

Fig. 20.1 Verbal intelligence and memory scores improve with age for the prematurely born. Serial evaluations of 90 very low birth weight preterm infants and 58 term control subjects: (*a*) the Peabody Picture Vocabulary Test (PPVT)-Revised verbal intelligence scores and (*b*) WISC III Digit Span raw scores. The results indicated that scores for the preterm group continue to improve over time (PPVT, group-by-time $p < 0.01$; digit span, group-by-time $p = 0.03$).

and/or myelination and intelligence measures in typically developing children (Aslin & Schlaggar, 2006; Pujol *et al.*, 2006), and significant brain–behavior relationships have been reported for prematurely born children as well. Inder *et al.* (2005) found that those preterm subjects with reductions in cortical and deep nuclear gray matter at term had moderate to severe neurodevelopmental disability at age 1 year, and both Woodward and Peterson (Peterson *et al.*, 2003; Woodward *et al.*, 2006) found that volume reductions correlated with neurodevelopmental delay at 18–20 months.

Furthermore, volume reductions in the temporal and sensorimotor regions have been shown to correlate with IQ scores in prematurely born subjects at age 8 years (Peterson *et al.*, 2000; Allin *et al.*, 2004). Lodygensky and colleagues (2005) found modest but significant correlations for preterm children at age 8 years between both hippocampal volumes and total cortical gray matter volume and WISC-R IQ scores. Kesler and colleagues (2006) reported that changes in left temporal gyrification correlated with reading skill in preterm children. Gimenez and colleagues (2006c) correlated thalamic volumes with verbal fluency in prematurely born children at age 14 years, and Allin and colleagues (2004) noted significant correlations between verbal IQ and verbal fluency scores with total mid-sagittal corpus callosum size and mid-posterior surface area for prematurely born males only at ages 14–15 years.

Finally, preterm subjects have been shown to have decreases in hippocampal volumes, and both Gimenez and colleagues (2004) and Isaacs and colleagues (2000) have reported correlations between hippocampal volumes and memory deficits in adolescents with a history of prematurity.

Functional imaging may explain improvements in language scores in the prematurely born

While MRI studies evaluating the cerebral volumetric sequelae of preterm birth have consistently reported alterations in those regions subserving language, the functional organization of auditory processing in prematurely born infants is just beginning to be explored (Fig. 20.2).

More functional MRI (fMRI) studies of language are available, however, for school-aged and older preterm subjects. Santhouse and colleagues (2002) tested the hypothesis that those abnormalities of the corpus

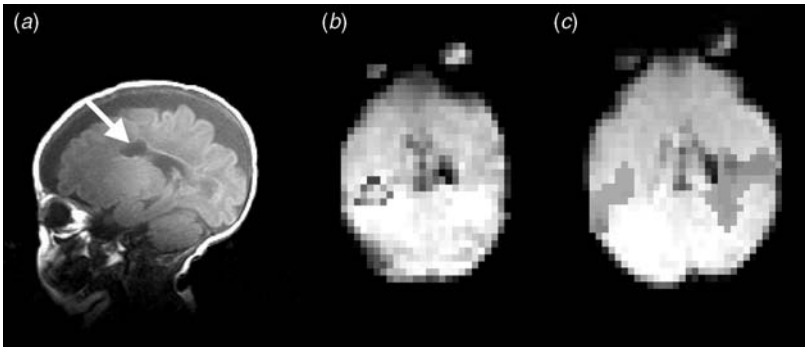

Fig. 20.2 (See color plate section.) Evidence that the functional magnetic resonance imaging (fMRI) BOLD response to auditory activation can change over time in a preterm infant with grade 4 intraventricular hemorrhage (IVH). This 24-week gestational age preterm infant with a left-sided grade 4 IVH and subsequent porencephaly (*a*; arrow) was first studied with echoplanar fMRI using a frequently modulated pure tone at term equivalent age; only right-sided BOLD signal in response to stimulus was detected (*b*) at that time. Six weeks later (*c*), bilateral auditory activation was detected using the same experimental paradigm. (Courtesy of A. Anderson, Vanderbilt University.)

callosum commonly found in preterm children may have functional significance. Employing an auditory paradigm in which prematurely born young adults judged whether pairs of sounds were similar or di erent, they studied seven preterm subjects with thinning of the corpus callosum, nine preterm subjects with normal corpus callosal volumes, and seven term controls at 18–20 years of age. There were no significant di erences in handedness or intelligence between the three groups of study subjects but callosal thinning subjects had significant loss of volume in the splenium, anterior corpus callosum, genu, forceps major, and forceps minor. They also had significant atrophy of the superior temporal gyrus in both hemispheres. In response to an fMRI auditory paradigm requiring callosal transfer of information, preterm subjects with thinning of the corpus callosum showed significantly greater activation in the right superior temporal gyrus (Brodmann's area [BA] 22) when compared with either group of controls ($p = 0.05$ for term controls; $p = 0.04$ for preterm controls), while no significant di erences were seen between the full-term and preterm control groups, suggesting that this group of prematurely born subjects had developed alternative neural strategies to compensate for the (presumably) perinatal structural damage.

Phonological processing skills are critical for the acquisition of skilled reading, and impairments in phonological processing have been repeatedly shown to contribute to the need for special educational services at school age. Peterson and colleagues (2002) compared brain activity associated with the phonological and semantic processing of language using a passive listening task in 26 preterm subjects and 13 matched term control children at age 8 years, and IQ was assessed using a standard measure of intelligence. During phonological processing, term control subjects deactivated extensive regions of the prefrontal cortex including BA 10, 46, and 47 as well as exhibiting negative blood oxygen level dependent (BOLD) signal in the ventral anterior cingulum (BA 25). During semantic activation, the term controls were found to have positive BOLD signal in both the inferior frontal gyri (Broca's region) and the superior temporal gyri (Wernicke's region) bilaterally. In contrast, the pattern of brain activity identified in the semantic processing task for the preterm children closely resembled the pattern of brain activity identified in the phonological processing task in the term control children, suggesting aberrant processing of language for the preterm group. The greater this resemblance in the preterm children, the lower their verbal comprehension IQ scores and the poorer their language comprehension during the scanning task.

Because language testing scores for prematurely born subjects have been shown to improve over time, we used the same passive listening task to test the hypothesis that subjects who were born prematurely would develop alternative systems for processing language (Ment *et al.*, 2006). In contrast to our report of children at age 8 years, we studied only preterm children who had both no known ultrasonographic evidence for neonatal brain injury and normal ventricular size at age 12 years; the results from 14 preterm subjects were compared with ten matched term controls, and all subjects were tested with the Clinical Evaluation of Language Fundamentals (CELF) and portions of the Comprehensive Test of Phonological Processing (CTOPP). The CELF measures the ability to understand spoken language, or semantic processing, while the CTOPP measures phonological processing in children and young adults.

Although there were significant differences in CELF scores between the subjects born prematurely and control subjects, there were no significant differences in the phonology tests between the two study groups. Both preterm and term children deactivated the left inferior parietal lobule and right inferior frontal gyrus during semantic processing, but in both cases the deactivation was significantly greater in the term subjects compared with that in the preterm group. Similarly, although both groups activated the left medial temporal gyrus, left angular gyrus, and posterior cingulate gyrus during semantic processing, the BOLD signal changes were significantly greater for the term control subjects.

In contrast to maps for semantic processing, the preterm subjects exhibited strongly positive BOLD signals in the left medial temporal gyrus, the right anterior medial temporal gyrus, and the left parahippocampal gyrus, whereas the term control subjects demonstrated widespread frontal and occipital deactivation during phonological processing in this passive listening task. When the data were adjusted for verbal IQ and age at scan, the preterm subjects exhibited strongly positive BOLD signals in the left medial temporal gyrus/superior temporal gyrus, left parahippocampal gyrus, posterior cingulate gyrus, and the right anterior medial temporal gyrus, whereas in these four regions a negative BOLD signal was found for the term control subjects. In both the left and right occipital gyrus, the term subjects demonstrated significant deactivation, whereas the preterm subjects exhibited mixed deactivation/activation signals. Finally, although both groups deactivated the anterior cingulate gyrus during the phonological subtraction for this passive listening task, the adjusted negative BOLD signal was far greater in the term group. These data suggest that children born prematurely engage different networks for phonological processing but have no differences in phonological testing scores from matched term control subjects.

Similarly, Rushe used fMRI to examine the impact of perinatal damage to the corpus callosum on phonological processing in young adults who were prematurely born (Allin *et al.*, 2004). Phonological processing is preferentially disrupted by damage to the developing corpus callosum, and Rushe hypothesized that young adults born preterm and with evidence of callosal thinning would display incomplete lateralization of language function to the left hemisphere. Rushe studied six male VLBW subjects with thinning of the corpus callosum and compared them with term controls at age 18 years. During the "ON," or phonological, condition of the fMRI task, subjects were visually presented with pairs of nonwords and asked to press a key when the pair of pseudowords rhymed. During the "OFF" condition, which alternated with the rhyming task, subjects were required to make letter case judgments of visually presented pairs of consonant letter strings (orthographic processing).

The BOLD signals for phonological processing in the control group in Rushe's study are consistent with previous studies that have explored phonological processing of visually presented stimuli in typically developing children and adults. Activation was seen in the left inferior temporal gyri and superior frontal cortex as well as in the left medial temporal gyrus, precentral gyrus, and putamen. Activation was seen bilaterally in the precuneus, the peristriate cortex, and the insula and was also found in the right cingulate and posterior cingulate gyrus. These data confirm that, even in normal right-handed male subjects, processing of visual phonological stimuli is not limited to the left hemisphere.

Despite no difference in task performance scores for the two groups, compared with the control group, the very preterm group showed a significantly reduced BOLD response in the left peristriate cortex, the left cerebellum, and the right precuneus during phonological processing. In addition, significantly increased activation for the preterm group compared with the controls was noted in the right precentral gyrus and superior frontal cortex, suggesting the development of compensatory systems, or "hyperfrontality" in the preterm brain.

Factors influencing outcome

Both biological and environmental factors affect the neurodevelopmental outcome of low birthweight preterm infants (Hack *et al.*, 1995; Pinto-Martin *et al.*, 1999; Vohr *et al.*, 2003). While previous studies suggest that increasing years of maternal education, a two-parent household and early intervention were associated with higher testing scores, recent molecular studies suggest the importance of gender and genetics when assessing the impact of injury to the developing preterm brain.

Early intervention

Both preclinical and clinical studies suggest the importance of early intervention for brain development and the acquisition of cognitive skills in the prematurely born (Faverjon *et al.*, 2002). A multicenter randomized

trial, undertaken by the Infant Health and Development Program, assessed the e ect of intensive educational enrichment on preterm infants. The program increased the IQ scores at years of age, less marked in infants with a birthweight less than 1000 g. Subsequent follow-up at 8 years of age showed only a marginal e ect on cognitive status, school achievement, or behavior (McCarton *et al.*, 1997).

Similarly, our analysis of 296 preterm infants serially tested from 3 to 8 years of age, considering a di erence in PPVT-R as the end point, revealed that early intervention had a significant e ect only when the mother had less than high school education (Ment *et al.*, 2003). Finally, Als and colleagues (2004) demonstrated improvement in both DTI fractional anisotropy (FA) values and behavioral testing in preterm infants who were exposed to an early intervention program instituted before they were even due to be born.

Maternal education

Almost 25 years ago, Cohen and Parmelee (1983) evaluated a cohort of 100 preterm infants and demonstrated that social factors including maternal education were more important than any other set of factors in relating to the child's cognitive performance at age 5 years. Smith and colleagues (1996) studied 212 high- and low-risk preterm infants and 128 full-term infants from low socioeconomic homes and high levels of maternal attention were positively related to infant development for all groups. Similarly, Goldenberg demonstrated that higher maternal language skills and a positive home environment were directly related to IQ in preterm infants at age 5 years (Goldenberg *et al.*, 1998), and Dammann *et al.* (2003) showed that maternal education was significantly associated with nonverbal IQ scores <70 in prematurely born children at nine years of age. Finally, using the Infant Health and Development Program cohort, Brooks-Gunn (McCarton *et al.*, 1997) showed that only preterm children whose mothers had less than a high school education benefited from early intervention, and Wocadio and Rieger (2006) demonstrated that resource utilization by preterm subjects at age eight years was not related to gestation in neurologically normal children, but rather to maternal education.

Gender

IVH, cerebellar hemorrhage, and white matter injury are more common in preterm males than female preterm neonates (Synnes *et al.*, 2001; Rutherford *et al.*, 2004; Limperopoulos *et al.*, 2005a; Tioseco *et al.*, 2006). Reiss *et al.* (2004) showed that the white matter loss at 8 years of age in children born prematurely was significantly more in males. The anatomical distribution of white matter loss was also gender specific, with males having predominantly temporal and females having predominantly deep white matter loss. There is also a gender-specific response to treatment. We have previously shown with a multicenter trial that early low-dose indometacin treatment decreased both the incidence and severity of IVH in VLBW preterm infants. Analysis of this cohort by sex showed that in males indometacin halved the incidence of IVH, eliminated parenchymal hemorrhage, and was associated with higher verbal scores at 3–8 years (Ment *et al.*, 2004). Finally, long-term outcome is also worse in premature males. Hack and colleagues (2002) showed that preterm males are less likely to go to college, and Saigal and colleagues (2006) showed that preterm males attained a significantly lower educational status and were less likely to be employed.

Since FA, a measure of white matter organization, has been shown to correlate with Wechsler full-scale IQ scores in term children (Schmithorst *et al.*, 2005), we tested the hypothesis that values for FA in preterm subjects would di er from those of term controls. This recent microstructural study of 29 preterm ELBW adolescents without neonatal brain injury and normal clinical MRI studies and 22 matched controls demonstrated that FA was lowest in the right inferior frontal gyrus in preterm males and that FA in that region correlated with cognitive outcome at age 12 years (Constable *et al.*, 2008).

The underlying mechanisms for this gender-specific di erential response to injury are not known. The classic view of di erential sexual development assumes that gonadal steroid hormones are responsible for behavior and testosterone and its metabolites promoted a masculine type of development of brain and behavior. Interestingly, however, preclinical studies have shown that that sexually dimorphic gene expression in the brain precedes gonadal di erentiation (Dewing *et al.*, 2003). There is also experimental evidence that the response of brain and glial cells to injury di ers among both sexes (Du *et al.*, 2004), and oligodendrocyte progenitors, the presumed target cells of white matter damage in the premature infant, were shown to proliferate better in female than in male oligos in vitro (Marin-Husstege *et al.*, 2004). Additionally,

the turnover of myelin is greater in female mice than in male mice (Cerghet *et al.*, 2006).

Genetics

Traditionally injury to the premature brain has been considered to result from interrupted development and detrimental e ects of aggressive neonatal intensive care. The role of causative or permissive genetic factors on the development of gestational-age-dependent disorders is not clearly defined. A multicenter retrospective study of 450 twin pairs born prematurely at a mean gestational age of 29 weeks and who had a mean birthweight of 1286 g documented the contribution of familial factors to IVH susceptibility but failed to show a significant genetic contribution for IVH (Bhandari *et al.*, 2006).

A recent association was shown in familial porencephaly. An autosomal dominant mode of inheritance with both variable expression and reduced penetrance has been reported in familial porencephaly cases (Zonana *et al.*, 1986). In family studies porencephaly segregated with collagen IV-a1 gene mutations on chromosome 13 (Breedveld *et al.*, 2006). Subsequently a mouse model with a semidominant mutation in the mouse *Col4a1* gene proved that the defect resulted in stroke both in the fetus, neonate, and adult. Despite this convincing evidence, no preceding hemorrhages were documented in infants with porencephaly. The same study also showed that *COL4A1* mutations (causing inhibition of collagen IV secretion and resulting in structural defects in the basement membrane) predisposed the carriers to a higher risk of intracerebral hemorrhage at birth and during adulthood (Gould *et al.*, 2005, 2006).

Genome-wide studies for genetics of premature brain damage are extremely limited and much of the work on the e ect of genetic factors has come from studies with candidate gene approaches. Cytokine gene polymorphisms have been of particular interest, as these are believed to be critical in the pathogenesis of neonatal brain damage. Such functional cytokine gene variants may result in increased or decreased production of pro- or antiinflammatory cytokines. Several authors also studied the association of tumor necrosis factor α-308 with IVH, and the results have been contradictory: Adcock and colleagues (2003) showed an increased risk for IVH, which was later contradicted by Heep and colleagues (2005). Harding and colleagues (2004) studied the interleukin (IL)-6-174 CC genotype, which may result in enhanced production of IL-6, in 130 preterm neonates and found that this genotype was associated with significantly greater incidence of white matter disease, grade 4 IVH, and higher rate of disability at 2–5 years of age. In contrast, Yanamandra and colleagues (2005) studied IL-10-1082G/A polymorphism and found no association with IVH or PVL.

Polymorphisms of genes associated with coagulation were also analyzed for an association with neonatal brain damage. The 34Leu polymorphism of the factor XIII gene is associated with a low rate of brain infarction and a higher incidence of primary intracerebral hemorrhage in adults. Gopel and colleagues (2002) studied 531 VLBW preterm neonates and 301 term controls for the e ect of this polymorphism for the development of isolated intracerebral hemorrhage or white matter disease and found that the factor XIII Val34Leu polymorphism protects preterm infants from white matter disease. Gopel and colleagues (2001) also studied the e ect mutations with a prothrombotic tendency, factor V Leiden and prothrombin G20210A in a cohort of 305 preterm VLBW infants. These authors found that the overall incidence of IVH was the same between infants with and without prothrombotic mutations. More recently, Hartel and colleagues (2006) studied 1195 preterm infants <1500 g birthweight and reported no di erence in the risk for grade 3–4 IVH with any of the following thrombophilic mutations: factor V Leiden, prothrombin mutation, factor VIII-323, and factor XIII Val34Leu.

Conclusions

The neurobehavioral sequelae of preterm birth is one of the major pediatric public health questions of our time, and emerging data suggest improvement in cognitive and behavioral skills in the prematurely born. Language and memory scores do not significantly di er between preterm and term control subjects at adolescence, but both structural and microstructural studies suggest marked changes in frontotemporal language systems in VLBW preterm groups. For these reasons, serial neurodevelopmental testing concurrent with functional imaging of well-described preterm cohorts may provide insight into the neurobiological mechanisms supporting recovery from injury in the prematurely born. Finally, experience and function can alter structure in the developing brain, and recent data suggest that intervention strategies may alter function as well (Duncan *et al.*, 1983). As neuroscientists probe connectivity and the genetic substrates of brain

development, it will be of both clinical and neurobiological importance to investigate the adaptive mechanisms of developing preterm brain.

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Chapter 21

Development of motor functions in health and disease

Mijna Hadders-Algra and Hans Forssberg

Theoretical framework of motor development

During recent decades knowledge on mechanisms governing motor control has rapidly increased. The expansion in knowledge was brought about by the development of sophisticated physiological, neurochemical, and imaging techniques. The idea that motor behavior was largely controlled by reflex mechanisms was abandoned. Nowadays, motility is regarded as the net result of the activity of complex spinal or brainstem machineries, which are subtly modulated by segmental a erent information and ingeniously controlled by supraspinal networks (Grillner *et al.*, 1995, 2005). Functional imaging makes it increasingly clear that supraspinal activity is organized in large-scale networks, in which cortical areas continuously interact with intermediary cortical or subcortical (striatal, cerebellar) structures (Hikosaka *et al.*, 2002; Molinari *et al.*, 2002). In the light of motor development it is good to realize that, in particular, cortical-subcortical networks expanded during phylogeny and that these networks determine to a large extent human motor ontogeny. This does not, however, preclude developmental changes in the spinal cord and muscles: developmental changes in one part of the neuromuscular system a ect those in other parts of the system.

Concurrent with the changes in insight into the neural mechanisms involved in motor control, knowledge on motor development has also increased – although at a considerably slower pace. This has led to changes in theoretical frameworks of the processes involved in the development of motor control. Initially, motor development was basically regarded as an innate, maturational process, but gradually it became clear that motor development is also a ected considerably by experience. The neuronal group selection theory (NGST) is a recently developed theory allowing equally prominent roles for genetic endowment, epigenetic cascades, and experience, and thus might o er an appropriate framework for the understanding of motor development (Edelman, 1989).

This chapter will start with an outline of what NGST might mean for the understanding of typical and atypical motor development. Next we will discuss typical and atypical motor development in the light of available data on neurodevelopmental mechanisms. We focus on the early phases of motor development.

The neuronal group selection theory

NGST and typical motor development

According to NGST, motor development is characterized by two phases of variability: primary and secondary variability (Hadders-Algra, 2000a). The borders of variation are determined by genetic information; genetic instructions are the major driving forces behind the functional topography of the human brain (Krubitzer & Kaas, 2005). During the phase of primary variability, motor behavior is characterized by abundant variation. The variation is brought about by explorative activity of the nervous system: the system explores all motor possibilities. The exploration generates a wealth of self-produced a erent information, which in turn is used for further shaping of the nervous system. Initially, however, the a erent information is not used for adaptation of motor behavior to environmental constraints. In other words, the phase of primary variability is characterized by variable but nonadaptive motor behavior (Hadders-Algra, 2000a).

At a certain point in time, the nervous system starts to use the a erent information produced by behavior and experience for selection of the motor behavior that fits the situation best, and the phase of secondary or

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adaptive variability starts. The selection process is based on active trial-and-error experiences. Indeed, evidence is accumulating that self-produced sensorimotor experience plays a pivotal role in motor development (Bertenthal *et al.*, 1994; Higgins *et al.*, 1996).

The transition from primary to secondary variability occurs at function-specific ages. For instance, in the development of sucking behavior, the phase of secondary variability starts prior to term age, and in the development of foot-placing during walking, it starts between 12 and 18 months (Hadders-Algra, 2000a). Around the age of 18 months all basic motor functions have reached the first stages of secondary variability. Due to the ingenious interaction between self-produced motor activities with trial-and-error learning and the longlasting developmental processes in the brain, such as dendritic refinement, myelination, and extensive synapse rearrangement (De Graaf-Peters & Hadders-Algra, 2006), it takes until the age of 18–20 years before the secondary neural repertoire has obtained its mature, adult configuration. In the adult situation human beings are equipped with a variable movement repertoire with an efficient motor solution for each specific situation.

NGST: motor development after a prenatal or perinatal lesion of the brain

A prenatal or perinatal lesion of the brain may be followed by atypical motor development, which may result in cerebral palsy or developmental coordination disorder (DCD). Note however that, in general, DCD cannot be attributed to a lesion of the brain (Hadders-Algra, 2002).

In terms of NGST, an early lesion of the brain has two major consequences (Hadders-Algra, 2000b). First, the repertoire of motor strategies is reduced. This results in less variable and more stereotyped motor behavior. Second, children with an early lesion of the brain have problems with the selection of the most appropriately adapted strategy out of the repertoire. The deficient capacity to select has a dual origin: it is related to deficits in the processing of sensory information and to the fact that the best solution may not be available due to repertoire reduction.

The practical consequences of the above-mentioned problems are twofold. First, the limited motor repertoire may result in the absence of a specific motor strategy, which would be available as the best solution in a specific situation for a typically developing child. As a consequence of the absence of the "best" solution, the child with an early lesion of the brain may have to choose a motor solution which di ers from that of the typically developing child. Thus, the di erent motor behavior of a child with cerebral palsy should not always be regarded as deviant, i.e., something which deserves to be "treated away," as it may be the child's best and most adaptive solution for the situation (Latash & Anson, 1996). Second, due to the deficits in the processing of sensory information, which hamper the process of selection of the best strategy, children with an early lesion of the brain need a ten-fold or hundred-fold more active motor experience than typically developing children (Valvano & Newell, 1998). As children with brain dysfunction need more practice than their non-a ected peers, it is important to reinforce the child's motivation by creating an ecological, playful setting with positive feedback.

Typical motor development

Nongoal-directed motor behavior

Prenatal motor behavior

A recent detailed ultrasound study on the emergence of fetal motility has revealed that the earliest movements can be observed at the postmenstrual age (PMA) of 7 weeks and 2 days (Lüchinger *et al.*, 2008). The first movements observed are slow, small sideways bending movements of head and/or trunk. A few days later, these simple movements develop into movements in which also one or two arms or legs participate. But the movements continue to be slow, small, simple, and stereotyped. At the age of 9–10 weeks' PMA general movements (GMs) – consisting of movements in which all parts of the body participate – emerge. Initially, GMs show little variation in movement direction, amplitude, and speed. But after a few days, the majority of GMs show a substantial degree of variation in speed, amplitude, participating body parts, and movement direction. The emergence of GMs with movement variation and complexity at 9–10 weeks' PMA coincides with the emergence of synaptic activity in the cortical subplate, which is the earliest maturing structure in the cortex (Kostovic & Judas, 2007). This coincidence and the finding that the evolution of the subplate neatly matches that of GM development (see p. 348) inspired the hypothesis that variable and complex GMs result from activity of the subplate modulating the basic

activity of the central pattern generator (CPG) networks of GMs in the spinal cord and brainstem (Hadders-Algra, 2007).

Soon after the emergence of the first movements, other movements are added to the fetal repertoire, such as isolated arm and leg movements, startles, various movements of the head (rotations, ante- and retroflexion), stretches, periodic breathing movements, and sucking and swallowing movements (Lüchinger *et al.*, 2008). The age at which the various movements develop shows considerable interindividual variation, but at about 16 weeks' PMA all fetuses exhibit the entire fetal repertoire. The repertoire continues to be present throughout gestation.

The amount of fetal motility varies enormously, both intra- and inter-individually (De Vries *et al.*, 1984). Still, some general characteristics can be distinguished. In the first place, GMs are the most frequently occurring movements (De Vries *et al.*, 1982; Roodenburg *et al.*, 1991). Second, fetal motility shows during the second half of gestation diurnal variation, with the lowest activity occurring in the morning and the highest in the evening (De Vries *et al.*, 1987). Third, the amount of motility decreases between 20 and 32 weeks' PMA. This reduction is due to a gradual decrease in the number of startles and stretches, and a sudden drop in the number of GMs between 28 and 32 weeks (Roodenburg *et al.*, 1991). According to Ten Hof and colleagues (2002), fetal motility continues to decrease slowly till term age, a decrease which cannot be attributed to developmental changes in fetal behavioral state. At present it is not clear whether the reduction in fetal motility can be explained by the increasing spatial limitation of the growing fetus in utero, or by intrinsic neurodevelopmental processes. The demonstration of a similar reduction in motility in low-risk preterm infants would be an argument in favor of the latter explanation, but at present the evidence available is conflicting (Prechtl *et al.*, 1979; Cioni & Prechtl, 1990).

The early presence of the fetal movement repertoire is intriguing. Spontaneously generated activity is a widespread phenomenon in the developing nervous system (O'Donovan, 1999) and may be regarded as an ontogenetic adaptation as it promotes further development of various organ systems, such as the nervous and musculoskeletal systems (Prechtl, 1984). For instance, it has been demonstrated that prevention of embryonic motility in the chick by blocking the neuromuscular junction results in a reduction of normally occurring neuronal death (Okada *et al.*, 1989) and in malformation of the joints (Oppenheim *et al.*, 1978) (cf. arthrogryposis multiplex congenita in human infants with impaired fetal motility). Analogously, fetal breathing movements are a prerequisite for proper lung development (Prechtl, 1981). Fetal motility can also be regarded as a preparation for postnatal life, the breathing movements forming a vital case in point (Hall & Oppenheim, 1987). It means that the newborn infant immediately at birth has a repertoire of motor behaviors that are necessary for survival. To this category of so-called "innate behaviors" belong feeding (i.e., rooting, sucking, mastication, swallowing), respiration as well as various protective reactions, such as blinking and coughing. There are also transient movement patterns which in general emerge during fetal life, such as infant stepping, which are present during some time after birth without any obvious meaning for the individual infant.

Postnatal motor behavior

At birth, be it term or preterm, minor changes in the motor repertoire occur. Breathing movements become continuous instead of periodic, the Moro reaction can be elicited for the first time, and the infant, who is now hampered by the forces of gravity, is no longer able to anteflex the head in supine position (Prechtl, 1984). In preterm infants the extrauterine environment also induces a change in the posture of the limbs – the intrauterine dominant flexion posture (Ververs *et al.*, 1998) changes into extension (Saint-Anne Dargassies, 1974). In preterm infants younger than 32 weeks, a dominant extension posture is present in both arms and legs. From 32 weeks onwards, the extension changes into a preference for flexion, at first in the legs and from about 36 weeks onwards also in the arms (Saint-Anne Dargassies, 1974). It should be realized, however, that: the age-dependent preference postures can only be observed during the relatively short periods with active wakefulness and not during sleep; and typical motor behavior is characterized by variation (Prechtl *et al.*, 1979; Cioni & Prechtl, 1990). With respect to the posture of neck and trunk, it has been reported that before 32 weeks' PMA antigravity postural control of the neck and trunk is entirely lacking (Saint-Anne Dargassies, 1974). During the following weeks some head control develops, so that at term age low-risk preterm infants, such as full-term infants, can keep the head upright for a few seconds while in a sitting position (Prechtl, 1977).

Around 36–38 weeks' PMA a transition in motor behavior can be observed. The quality of the GMs changes from the extremely variable "preterm" form into the more forceful "writhing" movements (Hadders-Algra, 2004), the fetus develops a head preference to the right side (Ververs *et al.*, 1994), and in the arms and legs a strong flexion posture becomes dominant (Saint-Anne Dargassies, 1974). The age of 36–38 weeks is also the age at which clearly defined behavioral states emerge (Nijhuis *et al.*, 1982), and the slow activity transient characteristics of the preterm electroencephalogram (EEG) disappear (Vanhatalo *et al.*, 2005; Vanhatalo & Kaila, 2006). Vanhatalo and co-workers (2005) suggested that these developmental changes might be related to the functional change of γ-aminobutyric acid (GABA) from excitatory to inhibitory (cf. Dupont *et al.*, 2006). However, the change in GABA function is not the only change in cortical neurotransmitter systems occurring near term; the glutamate system also exhibits developmental changes. During the periterm period, i.e., the period between 36–38 weeks PMA and 6–8 weeks post term, cortical glutamate receptors are transiently overexpressed (McDonald & Johnston, 1990; De Graaf-Peters & Hadders-Algra, 2006). It is conceivable that the glutamate receptor overexpression is the cortical correlate of the increased motoneuronal excitability observed during the periterm period (Hakamada *et al.*, 1988; Hadders-Algra *et al.*, 1992). The increase motoneuronal excitability around term age might explain why previously the motor behavior at this age was described as the phase of "physiological hypertonia" (Saint-Anne Dargassies, 1974). What could be the significance of this transient phase with increased motoneuronal excitability? It is conceivable that the high excitability of the motoneurons serves safeguarding of breathing movements, thereby assisting the transition from prenatal to postnatal life. This hypothesis is supported by the finding that problems in the continuation of respiration are especially encountered prior to 36 weeks (in the form of apneas [Prechtl *et al.*, 1979]) and around 3 months post term (in the form of the sudden infant death syndrome [Wennergren *et al.*, 1987]).

At the end of the temporary "writhing" phase another transformation in behavior takes place. The infant is significantly more awake than during earlier ages (Wol , 1984), and is able to use smiles and pleasure vocalizations in social interaction (Van Wul ten Palthe & Hopkins, 1984). Motor behavior also shows substantial changes. The GMs lose their "writhing" character and change into "fidgety" GMs, which consist of a continuous stream of tiny, elegant movements occurring irregularly all over the body (Hadders-Algra & Prechtl, 1992). The predominant flexor posture of arms and legs disappears, with the flexion decreasing somewhat earlier in the arms than in the legs (Touwen, 1976). At 2–3 months post term, the head can be stabilized on the trunk (Touwen, 1976), vestibular responsiveness has improved (Eviatar *et al.*, 1974), and steady visual fixation and brisk visual orienting reactions have been developed (Atkinson & Braddick, 1989). Functional neuroimaging studies suggest that an increasing activity in the basal ganglia, the cerebellum and the parietal, temporal, and occipital cortices could play a role in the transition at 2–3 months (Rubinstein *et al.*, 1989). Hadders-Algra and Prechtl (1992) demonstrated that the transformation of "writhing" GMs into "fidgety" GMs was more closely related to postmenstrual than to postnatal age. This suggests that endogenous maturational processes play a major, but not exclusive, role in this transition. The minor e ect of experience on the transition was indicated by Cioni and Prechtl (1990), who reported that low-risk preterms develop "fidgety" GMs about one week earlier than full-term infants.

The fact that the infant first becomes a socially attractive partner at the age of 2–3 months post term has induced speculations on the grounds of the typical term timing of human birth. Prechtl (1984) hypothesized that the "physiological preterm birth" of the human infant could be explained by the relative large brain and head size of the infant. A prolongation of gestation beyond term age would result in a further increase of brain and head size, thereby creating immense energetic and mechanical problems for the mother. Nature apparently preferred the term-birth solution with its transient phase of increased motoneuronal excitability.

Goal-directed motor behavior

Motor development during infancy

Goal-directed motor behavior develops during the final phase of general movement activity, i.e., during the phase of "fidgety" GMs. The emergence of voluntary motor behavior is announced by the ability of the infant to stop GM activity when it pays attention to something interesting. Next, goal-directed motility,

such as mutual manipulation of the hands, and general movement activity are displayed in an alternating fashion. With increasing age, general movement activity is gradually replaced by goal-directed behavior. In terms of neural networks this could mean that the generalized networks controlling GM activity are flexibly rearranged into multiple smaller networks, dedicated to the control of specific motor behavior, such as goal-directed motility of the arms and the legs, and postural control (cf. Simmers *et al.*, 1995).

Infant motor development is characterized by intra- and interindividual variation (Touwen, 1976, 1978; Hadders-Algra, 2000a). The variation occurs, for instance, as variation in the emergence of a function, variation in the performance of a function, variation in the duration of specific developmental phases, and variation in the disappearance of infantile reactions, such as the Moro reflex. The variation in development includes the co-occurrence of di erent developmental phases. For instance, infants of a certain age can alternate belly crawling with crawling on hands and knees (McGraw, 1943; Touwen, 1976). Healthy infants may also exhibit a temporary regression, an "inconsistency," in the development of a specific function (Touwen, 1976). As long as the regression is restricted to a single function, it can be regarded as another expression of developmental variation. The large variation in the attainment of milestones (Fig. 21.1) implies that the assessment of milestones has limited clinical value (Capute *et al.*, 1985). A slow development of a single function usually has no clinical importance, but, of course, the finding of a general delay is clinically relevant. For the assessment of motor milestones in preterm infants, the age of the infant should be corrected for prematurity, i.e., functional age should be calculated from term age onward (Touwen, 1980). This rule is especially useful during the first half year post term, when the variation in the emergence of motor skills lies in the order of about three months, which is equivalent to the degree of prematurity. At later ages the variation in motor skill attainment exceeds the degree of prematurity, thereby reducing the importance of age correction in older preterm infants.

Infancy is the period of transition of primary to secondary variability, i.e., from motor behavior that cannot be adapted to task-specific conditions to adaptive motor behavior. This transition occurs at function-specific ages. A recent observational study indicated that the transition in sitting behavior occurs between 6 and 10 months, that in abdominal progression between 8 and 15 months, that in reaching movements between 6 and 12 months, and that in grasping between 15 and 18 months (Heineman *et al.*, 2008). This means that after the transition infants could clearly visibly adapt motor behavior to the situation. When laboratory tools are used to study motor development signs of transition can be detected at earlier ages. For instance, EMG studies on the development of postural adjustments during sitting indicated that the transition from primary to secondary variability emerges after the age of 3 months (Hedberg *et al.*, 2005; De Graaf-Peters *et al.*, 2007), whereas the observational study indicated that it first starts at 6 months.

A major accomplishment during infancy is the development of postural control, resulting in the ability to stand and walk without support. Postural control is primarily aiming at the maintenance of a vertical posture of head and trunk against the forces of gravity, because a vertical orientation of the proximal parts of the body provides an optimal condition for vision and

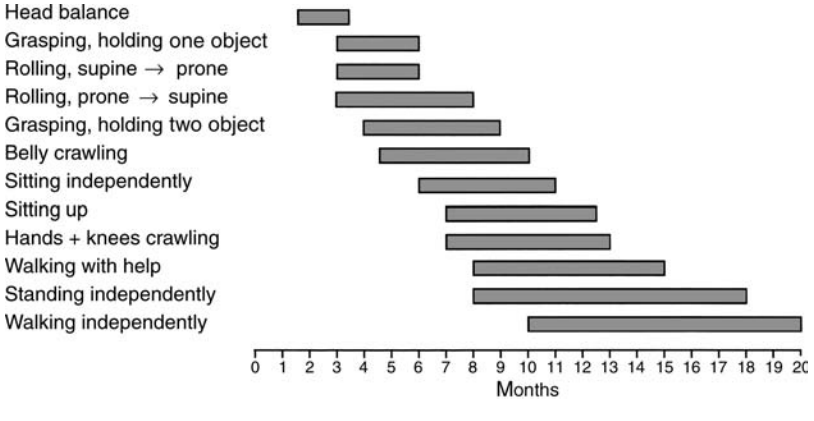

Fig. 21.1 Schematic representation of the ages at which some motor skills emerge during infancy. The length of the bars reflect the interindividual variation. (Adapted from Touwen, 1976.)

goal-directed motility (Massion, 1998). In the control of posture, two functional levels of control can be distinguished. The basic level of control deals with the direction-specificity of the adjustments: when the body sways forward dorsal muscles are primarily recruited, when the body sways backward the ventral muscles are primarily activated (Forssberg & Hirchfeld, 1994). The study of Hedberg and colleagues (2004) indicated that 1-month-old infants have direction-specific adjustments, which suggests that the basic level of postural control has an innate origin. Young infants show a variable repertoire of direction-specific adjustments from which, from the age of 4 months onward, they learn to select, by means of active trial and error, the adjustment that fits the situation best (Hadders-Algra, 2008). Meanwhile the infant learns to sit independently. With increasing age, the means to adapt postural activity become increasingly refined. A major developmental change is the emergence of anticipatory postural activity between 12 and 14 months, an ability which strongly promotes the development of independent walking (Hadders-Algra, 2008).

Successful reaching is preceded by various forms of prereaching activity. For instance, Von Hofsten (1982) demonstrated that newborn infants move their hands closer to a nearby object when they visually fixate it, than when they do not pay visual attention to the object. Reaching results in actual grasping of an object from 4 months onward (Touwen, 1976). At this age, reaching movements have an irregular and fragmented trajectory consisting of multiple movement units, which underlines the heavy reliance of the first reaching movements on feedback control mechanisms (Von Hofsten, 1979). During the following months, the reaching movement becomes increasingly fluent and straight, and the orientation of the hand is increasingly adapted to the object (Von Hofsten & Fazel-Zandy, 1984). After their first birthday, infants increasingly often use the pincer grasp to pick up tiny objects. This implies that corticomotoneuronal pathways are increasingly involved in fine motor control (Lemon *et al.*, 2004).

At birth, the infant – like the fetus – shows locomotor-like behavior in the form of neonatal stepping movements (McGraw, 1943; Prechtl, 1977). These movements are probably generated by spinal pattern generators analogous to the locomotion in the hindlimbs of kittens after a transection of the thoracic cord (Forssberg *et al.*, 1980), in spinal lampreys (Grillner *et al.*, 1995) or to the locomotor-like activity in people with a spinal cord injury (Van Hedel *et al.*, 2005). The infant stepping movements are rather primitive in character and di er largely from the flexible plantigrade gait of adulthood (Forssberg, 1985). The nongoal-directed neonatal stepping is characterized by a lack of segment-specific movements, implying that the legs tend to flex and extend as a single unit, by lacking a heel strike, a variable muscle activation with a high degree of antagonistic coactivation, and by short latency EMG bursts at the foot contact due to segmental reflex activity (Forssberg, 1985). In the absence of specific training, the stepping movements can no longer be elicited after the age of 2–3 months (Touwen, 1976). A period of locomotor silence follows, which is succeeded in the third quarter of the first postnatal year by goal-directed progression in the form of crawling and supported locomotion. When the neonatal stepping is trained daily, the stepping response can be elicited until it is replaced by supported locomotion (Zelazo, 1983). This is perhaps not so surprising considering that the locomotor pattern of supported locomotion is reminiscent of that of neonatal stepping – both lacking the determinants of plantigrade gait (Forssberg, 1985). Also the milestone transition into independent walking is not associated with a major change in specific locomotor activity. This indicates that the emergence of independent locomotion is not primarily induced by changes in the locomotor networks. Presumably, the development of independent walking is largely dependent on the development of postural control (Forssberg & Dietz, 1997).

Motor development during childhood

Motor development beyond infancy is characterized by a gradual increase in agility, adaptability, and the ability to make complex movement sequences. It is the phase of secondary variability, during which maturational processes in continuous interaction with experience produce highly adaptive secondary neuronal repertoires (Edelman, 1989; Hadders-Algra, 2000a). The creation of the secondary repertoires is associated with extensive synapse rearrangement, the net result of synapse formation, and synapse elimination (De Graaf-Peters & Hadders-Algra, 2006). It is facilitated by increasingly shorter processing times that can be attributed in part to ongoing myelination (Müller *et al.*, 1994).

Difficulties with the maintenance of balance dominate the picture of early locomotion. Postural control during balance-challenging conditions at toddler

age is achieved by an activation of all direction-specific leg, trunk, and neck muscles and a rather high amount of antagonistic coactivation (Hadders-Algra *et al.*, 1998). This "muscular corset strategy," which explains the stick-like locomotor behavior of the toddler, can be regarded as a smart initial solution of the nervous system to master the large degrees of freedom of the upright moving body (cf. Bernstein, 1935). But this energy-consuming en bloc strategy is only used till the age of 2.5–3 years (Hadders-Algra *et al.*, 1998). Thereafter, direction-specific postural behavior is adapted in increasingly sophisticated ways. The age-related improvements in postural control are reflected in the development of skills such as tandem gait, and standing and hopping on one leg, which emerge between 4 and 5 years and can be performed appropriately at 7–8 years. Typical for postural control prior to the age of 5–6 years is a stronger reliance on somatosensory information than on visual information. Between 5–6 years and 9 years postural control is in transition. During this transition various developmental processes take place: visual acuity and stereopsis reach adult values, the vestibular system obtains its maximum efficiency in the control of postural sway, children change from an egocentric reference frame to an allocentric one, and anticipatory postural behavior in stance is characterized by an overly active feedforward control. First, after the age of 9 years postural behavior starts to resemble adult postural behavior. But it takes at least until adolescence before real adult behavior has emerged (Hadders-Algra, 2008).

Locomotion obtains its plantigrade characteristics slowly in the period between 1 and 5 years (Forssberg & Dietz, 1997). The coactivation of the tibialis anterior and gastrocnemius muscles decreases from about 2 years onwards, due to a later occurrence of calf muscle activity in relation to tibialis activity. As a result, the heel strike develops. During this age period, the movements of the ankle become out of phase with those of the proximal joints. After 4 years of age, the reflex EMG activity at foot contact disappears, suggesting that walking is becoming controlled by increasingly complex polysynaptic circuitries (Berger *et al.*, 1984).

During the post infancy years, manual activities become increasingly complex. For instance, children learn to use eating utensils, to button their clothes, and to tie shoelaces (Gesell & Amatruda, 1947). The coordination of the precision grip changes considerably (Gordon & Forssberg, 1997). When the precision grip emerges, it lacks the adult coordination in parallel of the lifting load force and the squeezing grip force. Instead, 1-year-old children start with the generation of the squeezing grip force, while they simultaneously produce a negative load force by pressing the object to the support surface. A more in parallel force production emerges in the second half of the second year, but it is until about puberty before the perfect in parallel force coordination of the adult is accomplished. With increasing age, the ability to adapt grip forces to the slipperiness of the object develops. Also the ability to use anticipatory control mechanisms allowing the immediate scaling of grip forces to the known weight and the visually estimated size of an object is not immediately present. This ability emerges between 2 (known weight) and 3 years (visually estimated size) (Gordon & Forssberg, 1997).

Early damage of the brain and recovery of function

The young brain shows substantial plasticity. This plasticity explains why functional recovery after a lesion of the brain acquired during the prenatal or neonatal period in general is larger than that after similar lesions acquired during adulthood (Kennard, 1944; Armand & Kably, 1992). Functional recovery at early age is mediated by considerable anatomical reorganization in the form of excessive sprouting of fibers, increased dendritic arborization, maintenance of exuberant projections, including the persistence of ipsilateral corticospinal connections (Eyre, 2007), and lesion-induced synaptogenesis (Kolb & Whishaw, 1989). The extent of the recovery depends on a number of factors, such as the size and site of the lesion, and the age at which the insult of the brain occurred (Kolb & Whishaw, 1989; Staudt, 2007). The Kennard principle (Kennard, 1944) stating "the younger the age at insult, the better the outcome" is not always true. For instance, Villablanca and colleagues (1993) reported that lesions of the frontal cortex in fetal kittens resulted in neocortical dysgenesis and considerable sensorimotor deficits, whereas similar lesions acquired during the neonatal period were only followed by minimal functional deficits in the absence of gross structural anomalies. It is conceivable that lesions at very early age interfere with basic developmental processes in the brain, and consequently impede sensorimotor development to a larger extent than similar lesions occurring after the completion of these basic formative

processes – but still at a time that the nervous system is plastic and young. Not all anatomical reorganizations result in functionally efficient behavior. For instance, unilateral periventricular lesions – lesions which typically occur during the age period equivalent to the third trimester of pregnancy – may result in relatively good motor sparing due to the persistence of the ipsilateral corticospinal tract originating from the nondamaged hemisphere. But this is accompanied by a sensory processing system that remains in the damaged hemisphere, a configuration which could contribute to the inter-hemispheric sensorimotor integration problems often found in children with unilateral spastic cerebral palsy (Gordon *et al.*, 1999). Another example of mixed functional results after lesion-induced reorganization are the hindering mirror movements in some children with unilateral spastic cerebral palsy. Carr and colleagues (1993) provided evidence that relative sparing of independent finger movements in these children is mediated by an abnormal ipsilateral projection of corticospinal axons to the spinal cord at the cost of mirror movements (see also Vandermeeren *et al.*, 2003; Eyre, 2007; Staudt, 2007). Also Brouwer and Ashby (1991) found indications that the reorganization after brain lesion at an early age could add to later motor dysfunction. They studied the projections of cortical neurons activated by transcranial magnetic stimulation to the motor neurons of the lower limb muscles in children with cerebral palsy, and concluded that the abnormal projections from the motor cortex to the spinal motor neurons in the children with cerebral palsy could contribute to the impairment in voluntary motility.

Other factors which (seemingly) a ect recovery of function after brain lesion at early age are training and the age at which function is evaluated. With respect to the latter, it should be kept in mind that it can take a considerable time before specific functions, such as the precision grip, are fully developed. Consequently, it can be equally long before certain dysfunctions become apparent (Hadders-Algra, 2002). With respect to training: animal experiments indicate that specific training and enriched environments promote developmental outcome after brain lesion at an early age (Kolb & Whishaw, 1989). However, for the human little evidence is available that early intervention after a lesion of the brain at an early age promotes developmental outcome (Blauw-Hospers & Hadders-Algra, 2005). The most promising program before term age most likely is the Newborn Individualized Developmental Care and Assessment Program (NIDCAP; Als *et al.*, 1994), which aims at mimicking the intrauterine environment and teaching caregivers principles of newborn behavior. After term age intervention by means of specific motor training programs, such as training of locomotor movements on a treadmill, general developmental programs may exert a positive e ect on motor development. However, confirmation of this assumption is needed. Relevant in this context is the notion that no evidence exists that commonly used early intervention techniques such as NeuroDevelopmental Treatment or treatment according to Vojta have a beneficial e ect on motor development (Blauw-Hospers & Hadders-Algra, 2005).

Abnormal motor development

Early phases of abnormal motor development

The plasticity and developmental changes of the young brain hamper long-term prediction of motor outcome after brain lesions at early age. On the one hand, an early acquired brain lesion may not result in a motor disorder; on the other hand, it can take some time before motor dysfunction is expressed. In this respect, it is striking that the quality of spontaneous motility at young age, i.e., the quality of GMs, can predict motor outcome to some extent (Prechtl, 1990; Hadders-Algra, 2004). The basic parameters of GM quality are movement variation and complexity: in abnormal GMs movement complexity and variation are reduced or absent (Fig. 21.2) (Prechtl, 1977). Prediction on the basis of longitudinal series of GM assessments is best. Second best is prediction on the basis of an assessment at "fidgety" GM age, i.e., at 2–4 months post term. Definitely abnormal GMs at "fidgety" age, i.e., the presence of movements that are characterized by a virtual absence of variation and complexity and which lack the age-specific "fidgety" nature, are related to cerebral palsy (Hadders-Algra, 2004; Prechtl *et al.*, 1997). Mildly abnormal GMs at "fidgety" GM age characterized by an insufficient amount of variation and complexity are associated with minor neurological dysfunction and behavioral problems at school age (Fig. 21.3) (Hadders-Algra, 2004). Recently it has been postulated that abnormal GMs are the result of damage or dysfunction of the cortical subplate and its e erent connections in the periventricular white matter (Hadders-Algra, 2007). Of course, the assessment of the quality of GMs is only

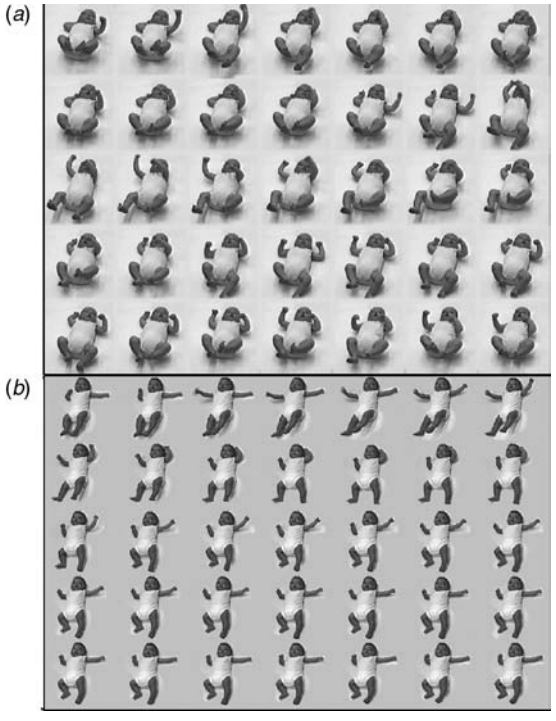

Fig. 21.2 Representation of video frames with general movements (GMs) of two infants at fidgety GM age. The video recordings start in the left hand upper corner and should be read as the lines in a book. The interval between the video frames is 0.24 seconds. The infant in (*a*) was born at term and shows normal fidgety GMs. The continuously varying positions of the limbs illustrate the rich spatial and temporal variation of normal movements. The infant in (*b*) was born at 28 weeks postmenstrual age. She shows definitely abnormal GMs. The abnormal character of the movement is reflected by the lack of variation, indicated by the virtually identical frames, which induce the false impression that the infant hardly moves. (Video recordings made in collaboration with the Department of Developmental and Experimental Clinical Psychology; figure published with permission of the parents and the Nederlands Tijdschrift voor Geneeskunde [Hadders-Algra, 1997].)

one of the clinical tools that may assist the prediction of developmental outcome. Other clinical tools that provide information on the risk of developmental dysfunction are the various forms of imaging of the young brain (e.g., ultrasound, traditional magnetic resonance imaging [MRI], di usion-weighted imaging), electrophysiological assessments (electroencephalography [EEG], visual and somatosensory evoked potentials), and the neurological examination (De Vries & Groenendaal, 2002; Majnemer & Snider, 2005).

Motor problems in the child with cerebral palsy

The cerebral palsies are a heterogeneous group of serious motor disorders due to damage of the immature brain, a ecting 1 in 500 infants (Himmelmann *et al.*, 2005). A central feature of cerebral palsy is impaired control of movement and posture. The motor impairment is often accompanied by impairment of sensation, cognition, communication, behavior, and by a seizure disorder (Bax *et al.*, 2005). The presentation of the motor impairment is also heterogeneous. The major motor dysfunctions in cerebral palsy are presented in Table 21.1. Basically, the motor dysfunctions of cerebral palsy can be divided into two categories. One category consists of pathological features, which are added to the motor behavior. These "extra" motor properties are in the (+) column in Table 21.1. These are the dysfunctions well known to clinicians, and are included in most textbook chapters on cerebral palsy. However, gradually awareness has grown that the motor behavior of children with cerebral palsy is also characterized by an improper coordination, indicating deficiencies of sensorimotor control. The problems in programming of motor behavior, which fit to the theoretical framework of NGST, can strongly interfere with movement production and may be as deleterious to motor activity as the "extra" (+) pathological motor properties. The central dyscoordination stands for a deficient neural control and reflects a failure to develop proper sensorimotor mechanisms. Therefore, it is marked with a (–) in Table 21.1. Likewise, paresis is under (–) since it is also due to a lack of proper neural control. In this context it represents a deficient capacity to produce a powerful muscle contraction during voluntary movements. The central dyscoordination and paresis can be due to damage of the same neural circuits, but the two functional phenomena and their neural substrates may also vary independently.

The development of cerebral palsy during infancy is primarily characterized by the lack of variation (Touwen, 1978). The lack of variation can be expressed as stereotyped motility and stereotyped postures, such a stereotyped flexion of the arms, fisting of the hands, extension of the legs, clawing of the toes, dominant asymmetrical tonic neck reflex (ATNR) posturing, hyperextension of neck and trunk, or stereotyped asymmetries (Touwen, 1978; Amiel-Tison & Gosselin, 2001; Heineman *et al.*, 2008). Other frequently occurring signs are a poor head balance, abnormalities in muscle tone, visuomotor signs, and persisting infantile reactions (Amiel-Tison & Gosselin, 2001). The motor dysfunctions are often associated with a delay in motor development.

Deficits in postural control play a dominant role in the motor problems of cerebral palsy. This holds especially true for children with severe bilateral spastic cerebral palsy and severe athetosis, who do not develop an adequate head balance and an ability to sit without help. There is some evidence that these profound postural deficits can be attributed to the absence of direction-specific postural activity, i.e., the basic form of postural coordination (Brogren Carlberg & Hadders-Algra, 2008). In children with less severe forms of cerebral palsy the basic level of postural organization is intact. But these children invariably have difficulties with the adaptation of postural adjustments to the situation. The problems in adaptation may be attributed in part to impaired processing of sensory information – an often encountered problem in children with cerebral palsy. The problems in adaptation may result in the use of alternative strategies, such as an excess of antagonistic coactivation to compensate for deficient fine-tuning mechanisms (Brogren Carlberg & Hadders-Algra, 2008; Woollacott & Crenna, 2008).

Independent walking in children with cerebral palsy is hambered not only by postural deficits, but also by specific locomotor dysfunctions. The locomotor pattern of children with cerebral palsy, who are able to walk (with help), is characterized by features mimicking immaturity, such as the persistence of coactivation of antagonistic leg muscles during the stance phase of gait, and a nonplantigrade gait. The phasic EMG amplitude is reduced, which probably reflects the clinical sign of paresis. In children with spastic forms of cerebral palsy, spasticity (the velocity-dependent increase of muscle response to imposed stretch) and secondary changes in the properties of the muscles contribute to the locomotor problems (Woollacott & Crenna, 2008).

Children with severe forms of cerebral palsy do not develop a precision grip, but children with milder forms do (Ingram, 1966). In the latter group, the force coordination during precision grip is inappropriately organized (Eliasson *et al.*, 1991). The children use an excessively large squeezing grip force during the static "in air" phase at the

Table 21.1 Motor dysfunctions in cerebral palsy

Functional surplus signs (+)	Functional deficits (–)
Spasticity	Paresis
Musculoskeletal deformations	Central dyscoordination
Dyskinesia	• Co-contractions
Hyperreflexia	• Mirror movements
Persisting developmental reaction	

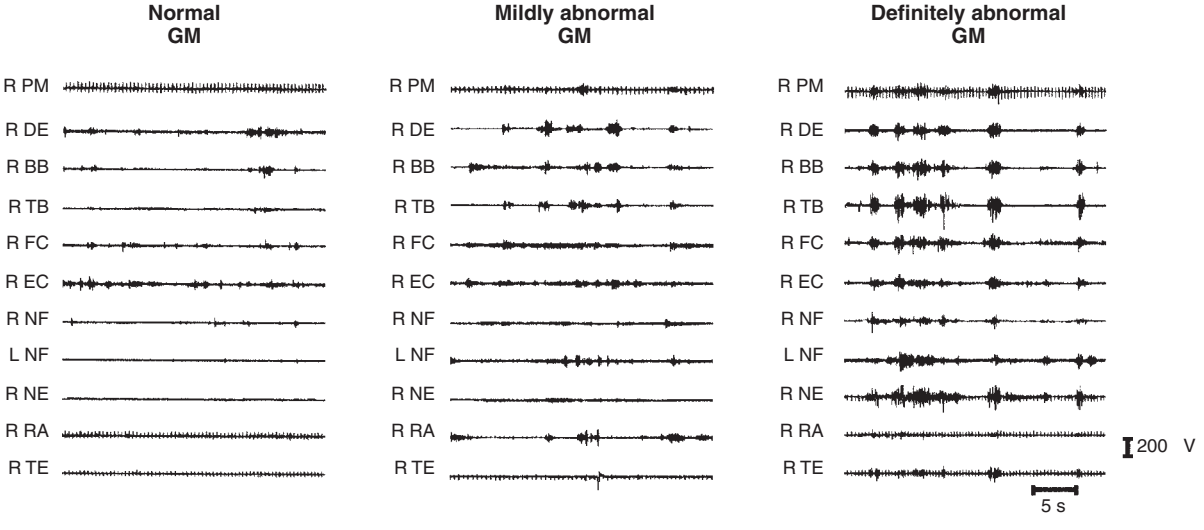

Fig. 21.3 Electromyographic (EMG) recordings of arm, neck, and trunk muscles during a normal, a mildly abnormal and a definitely abnormal general movement (GM) at 3 months of age. The normal EMG pattern shows the variable bursting pattern with small phasic bursts which is characteristic of normal "fidgety" GMs. The mildly abnormal pattern has conserved the variability in bursting, but the size (amplitude and duration) of the phasic bursts is disproportionally large. In the definitely abnormal pattern the variation in bursting is totally absent: the majority of recorded muscles are activated synchronously. L, left; R, right; BB, biceps brachii muscle; DE, deltoid muscle; EC, extensor carpi muscles; FC, flexor carpi muscles; NE, neck extensor muscles; NF, neck flexor muscles; PM, pectoralis major muscle; RA, rectus abdominis muscle; TB, triceps brachii muscle; TE, thoracic extensor muscles.

end of the object-lifting movement. Grip forces are poorly adjusted to the slipperiness of the surface of the object and anticipatory control mechanisms are lacking. The latter suggests that children with cerebral palsy have a diminished capability to build appropriate internal representations (Gordon & Forssberg, 1997).

Motor problems in the child with developmental coordination disorder

Children who have a marked impairment in the performance of functional motor skills in the absence of clear neurological pathology nowadays are classified as having DCD (American Psychiatric Association, 1994). DCD has a prevalence of 5%–10% and is expressed heterogeneously. Children with DCD may exhibit impaired fine motor, gross motor, or visuomotor skills, either as a single problem or as a combination of impairments (Geuze *et al.*, 2001). Relatively little is known about the etiology of DCD, even though it is recognized that preterm children exhibit DCD more often than their term peers (Holsti *et al.*, 2002). The neurological makeup of children with DCD may provide a clue as to whether prenatal or perinatal adversities contribute to the development of DCD (Hadders-Algra, 2002). Recently two forms of minor neurological dysfunction (MND) have been distinguished: simple and complex MND. The distinction is based on age-specific criteria. Prior to the onset of puberty the distinction is based on the number of signs of dysfunction – children with complex MND exhibit more dysfunctions than those with simple MND; after puberty the distinction is based on the type of dysfunction. Simple MND is present in about 20% of the population and may be regarded as the expression of a normal but nonoptimally developed brain. Actually it would have been better if the D in simple MND denoted not dysfunction but di erence. Probably a major part of simple MND may be attributed to genetic constitution, but simple MND is also associated with "perinatal stress," i.e., intrauterine growth retardation, uncomplicated prematurity, or mild asphyxia. In contrast, complex MND is the form of MND that is strongly related to prenatal and perinatal adversities and to DCD, learning, and behavioral disorders, such as attention-deficit hyperactive disorder (ADHD). It has been suggested that it may be considered as a borderline form of cerebral palsy. Complex MND is found in 3%–5% of children.

Little is known about the pathophysiological mechanisms underlying the motor problems of children with DCD, probably due to the heterogeneity of the children evaluated at school age. Deficits in the processing of sensory information may play a role. Problems in visuospatial processing are most frequently reported, and deficits in visuoperceptual and visual memory ability and in kinesthetic perception are reported less often (Wilson & McKenzie, 1998). The data on motor organization are diverse. Nevertheless, features most often reported in children with DCD are relatively slow and more variable movements and impairments in temporal adaptation and force coordination (Pereira *et al.*, 2001; Jucaite *et al.*, 2003). The elegant study of Lundy-Ekman and coworkers (1991) on hand motor control in children with DCD demonstrated that an increased variation in the timing of motor events was specific for children with mild coordination problems, whereas an increased variation in force output was characteristic for children with minor neurological signs suggestive of basal ganglia dysfunction, i.e., signs such as the presence of choreiform or athetotiform movements. The Lundy–Ekman paper underscores the notion that studies on motor mechanisms in children with DCD should take into account the type of the child's motor dysfunction.

Concluding remarks

Gradually it has become clear that variation and selection are fundamental features of typical motor development. Damage of the brain at an early age may result in reduction of motor repertoires and impaired selection of appropriate motor strategies, thereby interfering with adaptation of motor behavior to task-specific conditions. Deficits in the processing and integration of sensory information play a role in the impaired adaptation. Knowledge about the exact nature of developmental motor dysfunctions is growing but is still relatively poor. Additional information is urgently needed, as it o ers the basis for therapeutic intervention.

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Chapter 22

Antenatal glucocorticoids and programming of neuroendocrine function and behavior

Stephen G. Matthews and Amita Kapoor

Introduction

Glucocorticoids (cortisol in humans and most mammals, and corticosterone in rats, mice, and other lower vertebrates) are essential for normal brain development. They exert a wide spectrum of effects in most regions of the developing brain, ranging from subcellular reorganization to neuron–neuron and neuron–glial interaction. However, sustained elevation in, or removal of these hormones from, the fetal brain is detrimental to these processes, and can permanently modify the structure and function of the brain (Charmandari *et al*., 2003).

The pioneering work of Liggins and Howie in the early 1970s led to the widespread use of synthetic glucocorticoids to treat fetuses at risk of preterm delivery (Liggins & Howie, 1972). Preterm delivery occurs in approximately 7% of pregnancies in North America and results in approximately 75% of all neonatal deaths. In such cases, antenatal synthetic glucocorticoid therapy, administered between 24 and 34 weeks' gestation is highly effective in reducing the frequency of respiratory complications and perinatal death (National Institutes of Health [NIH] Consensus Panel, 1995). In 1994, the NIH endorsed the use of maternal synthetic glucocorticoid therapy during pregnancy (NIH Consensus Panel, 1995) and a similar approach was adopted around the world. Due to difficulty in predicting preterm birth, many women received repeat courses of synthetic glucocorticoids. Indeed, surveys in the late 1990s revealed that repeated dosing had become common practice in the late 1990s, with some women receiving up to 11 repeat courses (Brocklehurst *et al*., 1999). At the same time, data from animal studies were beginning to demonstrate long-term effects of fetal synthetic glucocorticoid exposure on endocrine function and behavior (O'Regan *et al*., 2004). In 2000, an NIH consensus update conference recommended limiting multiple course synthetic glucocorticoids to ongoing clinical trials (Committee on Obstetric Practice, 2002). As a result, there has been a move away from multiple course therapy. However, large prospective trials comparing the efficacy and safety of single versus multiple course therapy on neonatal outcome are being undertaken (Multiple Antenatal Corticosteroids [MACS], Toronto) or have been recently completed (Australian Collaborative Trial [ACTORDS]). Importantly, the latter study reported that repeat course synthetic glucocorticoids improved short-term neonatal outcome compared with single course therapy, and concluded that pending long-term outcome results, the short-term benefits support use of repeat doses of synthetic glucocorticoids (Crowther *et al*., 2006).

Long-term follow up of children and adults following antenatal synthetic glucocorticoid exposure are now beginning to be reported. A follow-up study of 30-year-old men and women failed to identify overt neurological or physiological effects of a single course of synthetic glucocorticoid therapy in late gestation (Dalziel *et al*., 2005). However, early markers of insulin resistance were associated with single course synthetic glucocorticoid exposure. Retrospective studies have also been undertaken. A recent study identified hyperactivity and attentional disorders but no effects on intelligence in 3- and 6-year-old children who had been exposed to repeated antenatal synthetic glucocorticoid therapy, compared with those who had been exposed to a single course (French *et al*., 2004). Therefore, evidence does suggest that while multiple course synthetic glucocorticoid therapy may benefit

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neonates in terms of lung function, there is also an increased risk of other negative outcomes, including long-term effects on behavior.

In addition to use in cases of preterm labor, synthetic glucocorticoids are also administered to pregnant women in other clinical situations including congenital adrenal hyperplasia. Often these treatments occur in early pregnancy and in some cases throughout gestation (Hirvikoski *et al.*, 2007). There is currently very little information as to the impact of such treatment regimens on brain and neuroendocrine development. However, a recent retrospective study has reported long-term effects on verbal working memory and on certain aspects of self-perception in children and adolescents (7–17 years of age) who had been exposed to such a regimen of antenatal synthetic glucocorticoids (Hirvikoski *et al.*, 2007).

Although the acute and long-term effects of synthetic glucocorticoids on neuroendocrine function and behavior are the focus of this chapter, fetal exposure to excess glucocorticoids can occur via increased maternal anxiety during pregnancy. In this connection, increased maternal anxiety and elevated maternal salivary cortisol levels during pregnancy have been associated with an increased incidence of behavioral problems in childhood (O'Connor *et al.*, 2003).

Development of the brain and neuroendocrine systems

Within the developing brain, the limbic system (primarily the hippocampus) is particularly sensitive to endogenous and exogenous glucocorticoids during development. The hippocampus has a myriad of complex functions within the brain. These include cognition, behavior, memory, coordination of autonomic activity, and regulation of a number of endocrine systems (de Kloet *et al.*, 2005). Given the wide spectrum of regulatory roles, programming of limbic function during development will have a profound impact in postnatal and adult life. Indeed, it has been known for four decades that hypothalamo–pituitary–adrenocortical (HPA) function can be permanently programmed during development, although the mechanisms have remained unclear.

The hypothalamic paraventricular nucleus (PVN) controls pituitary–adrenocortical activity (see Fig. 22.1). The limbic system, primarily via the hippocampus, forms a major inhibitory input to the PVN. Parvocellular neurons in the PVN synthesize corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which are then released into the hypophysial portal circulation (Herman *et al.*, 1996). CRH and AVP stimulate the release and synthesis of adrenocorticotrophin hormone

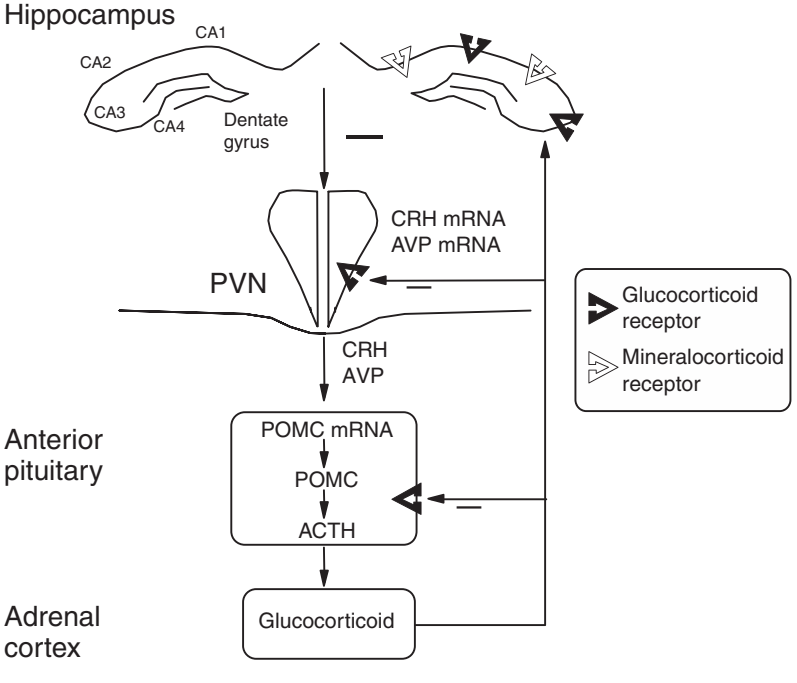

Fig. 22.1 A schematic representation of the hypothalamo–pituitary–adrenal (HPA) axis. The hypothalamic paraventricular nucleus (PVN) controls pituitary–adrenocortical activity. The limbic system, primarily via the hippocampus, forms a major inhibitory input to the PVN. Parvocellular neurons in the PVN synthesize corticotrophin-releasing hormone (CRH) and vasopressin (AVP), which are then released into the hypophyseal portal circulation. CRH and AVP stimulate adrenocorticotrophin (ACTH) synthesis and release, which then initiates the synthesis and secretion of cortisol from the adrenal cortex. Glucocorticoids feedback, via glucocorticoid and mineralocorticoid receptors, at several sites to inhibit further HPA activity. POMC, proopiomelanocortin.

(ACTH) from proopiomelanocortin (POMC) in the corticotrophs in the anterior pituitary gland (Herman *et al.*, 1996). ACTH then initiates the synthesis and secretion of cortisol from the adrenal cortex. Glucocorticoids act at multiple loci within the body not only to maintain homeostasis but also act in the central nervous system (CNS) to modify behavior and learning (Lupien & McEwen, 1997). However, extended tissue exposure to glucocorticoids is detrimental and as a result the HPA axis is tightly regulated (Charmandari *et al.*, 2003). Glucocorticoid feedback, via the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in the hippocampus and GR at the hypothalamic PVN and anterior pituitary, inhibits further HPA activity (Herman *et al.*, 1996; de Kloet *et al.*, 2005). Unlike endogenous glucocorticoids, synthetic glucocorticoids bind predominantly to the GR as the MR has low a nity for them (de Kloet *et al.*, 2005). Therefore, the effects on HPA axis development after antenatal synthetic glucocorticoid administration are likely mediated at the level of this receptor. In addition to binding to GR, synthetic glucocorticoids may also bind to neurosteroid receptors as well as in uence orphan receptors in the brain, including the pregnane-X-receptor (Kliewer *et al.*, 1998).

The timing of maturation of the HPA axis relative to birth is highly species specific, and is closely linked to landmarks of brain development (Dobbing & Sands, 1979). In animals that give birth to mature young (sheep, guinea pigs, and primates) maximal brain growth and a large proportion of neuroendocrine maturation (including corticosteroid receptor development) takes place in utero (Owen & Matthews, 2003). In contrast, in species that give birth to immature young (rats, rabbits, and mice), much neuroendocrine development occurs in the postnatal period (Sapolsky, 1996). Therefore, maternal glucocorticoid treatment in late gestation will impact on different stages of brain and HPA development depending on the species studied. Another important consideration when extrapolating between studies and species is that of receptor sensitivity. In this respect, mice and rats are corticosensitive (high receptor a nity for glucocorticoids) compared with other species such as guinea pigs and primates, which are considered corticoresistant.

Transplacental transfer of glucocorticoids

Under normal circumstances, and in all species studied, access of maternal endogenous glucocorticoids (cortisol and corticosterone) to the fetus is low. This is due, in part, to the expression of 11β-hydroxysteroid dehydrogenase (11β-HSD) in the placenta (Seckl & Holmes, 2007). Brie y, 11β-HSD interconverts cortisol and corticosterone to inactive products (cortisone, 11-dehydrocorticosterone). There are two known isoforms 11β-HSD type 1 which is bidirectional and type 2 which is uni-directional (cortisol to cortisone). The e ciency of placental 11β-HSD2 varies considerably among species, however it is generally accepted that placental 11β-HSD2 is of primary importance in excluding maternal glucocorticoids from the fetus (Seckl & Holmes, 2007).

Unlike endogenous glucocorticoids, synthetic glucocorticoids are not metabolized by placental 11β-HSD2. However, multidrug resistance P-glycoprotein (MDR P-gp), which was first discovered in drug-resistant tumor cells, is a 170-kDa membrane protein that removes substances (xenobiotics) from cells by adenosine triphosphate (ATP)-dependent extrusion. In humans, P-gp is encoded by a single *MDR1* gene, whereas in rodents, it is encoded by two genes; *mdr1a* and *mdr1b* (Yu *et al.*, 1993). P-gp is unique in that it can transport a range of structurally diverse compounds, including endogenous and synthetic glucocorticoids (Petropoulos *et al.*, 2007). Emerging evidence indicates that P-gp, which is expressed at high levels in syncytiotrophoblast cells of the placenta can act to reduce transplacental transfer of synthetic glucocorticoids from the maternal circulation to the fetus (Sun *et al.*, 2006). It has recently been shown that *mdr1* expression and P-gp activity decrease in the mouse, guinea pig, and human placenta in late gestation (Sun *et al.*, 2006; Petropoulos *et al.*, 2007). This would suggest that the ability of the placenta to exclude synthetic glucocorticoids from the fetus decreases in late gestation.

Acute effects of synthetic glucocorticoids on HPA development

Maternal administration of synthetic glucocorticoids during pregnancy acutely affects the developing fetal HPA axis at the level of the brain, pituitary and adrenal. Synthetic glucocorticoids enter the fetal circulation and attenuate fetal HPA axis activity through binding the GR in the fetal brain (Fig. 22.2). However, these effects are sex dependent (McCabe *et al.*, 2001; Owen & Matthews, 2003). (For a review of the normal profile of HPA axis development see Challis *et al.*, 2000.)

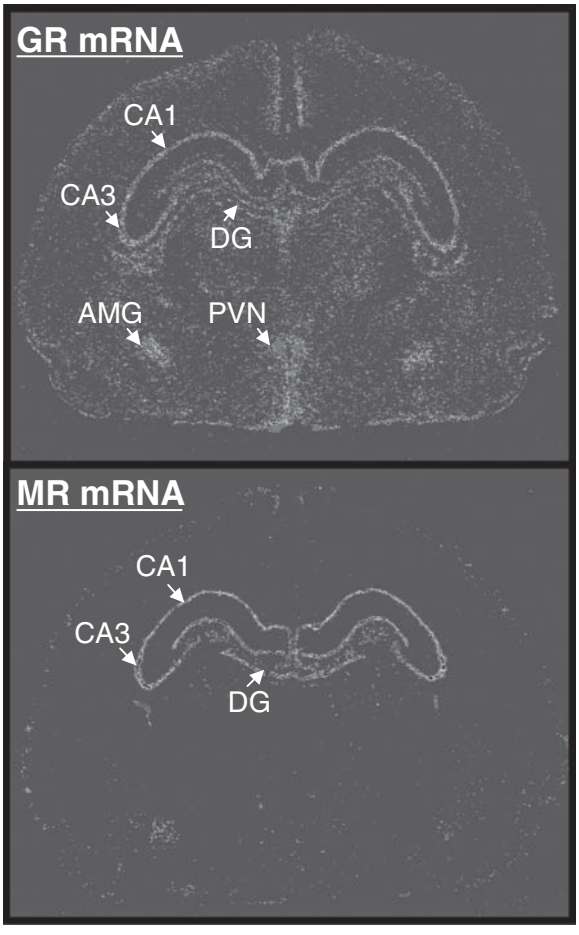

Fig. 22.2 (See color plate section.) Color-enhanced image illustrating the expression of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA in coronal sections of the fetal guinea pig brain. Receptor mRNA was determined by in situ hybridization using ^{35}S-labeled oligonucleotide probes speci c for GR and MR. GR mRNA is present at high levels in the CA1–CA4 elds of the hippocampus, the dentate gyrus (DG), paraventricular nucleus (PVN) and amygdala (AMG). Lower levels of GR mRNA are present in other brain regions. In contrast, MR mRNA is con ned almost exclusively to the limbic system. Red, high expression; yellow, moderate expression; green, low expression; blue, no expression.

In humans, maternal treatment with synthetic glucocorticoids leads to a reduction in cortisol measured in umbilical cord samples, taken soon after maternal treatment. However, due to minimal tissue availability, very little is known as to the direct actions of maternally administered synthetic glucocorticoids in the fetal brain. Nonetheless, GR and MR mRNA are present in the human hippocampus at 24 weeks of gestation. This is consistent with other long-gestation species (guinea pigs, sheep, and nonhuman primates) in which both GR and MR are present prior to birth (Noorlander *et al.*, 2006). There was no effect of maternal synthetic glucocorticoid exposure on hippocampal GR or MR expression in neonates ranging from gestational week 24 to 33 weeks (Noorlander *et al.*, 2006). However, the variability in synthetic glucocorticoid exposure and fetal age makes interpretation of these studies di cult.

In the fetal monkey, maternal treatment with the synthetic glucocorticoid dexamethasone results in prolonged suppression of fetal adrenocortical activity (Coe & Lubach, 2005). In baboons, maternal synthetic glucocorticoid administration of betamethasone (0.875 mg/kg) at 12-hour intervals from gestation day (gd) 121 to 135 (term ~185 days) greatly increased placental 11β-HSD2 mRNA and protein expression. Since this enzyme is vital in controlling the passage of endogenous maternal glucocorticoids to the fetus, betamethasone-induced upregulation of placental 11β-HSD2 expression may ultimately limit further fetal exposure to endogenous maternal glucocorticoids (Ma *et al.*, 2003).

In guinea pigs, which give birth to neuroanatomically mature young, administration of a single course of dexamethasone (1 mg/kg; two doses, 24 hours apart) to pregnant guinea pigs during the period of maximal brain growth (gd 50; term ~70 days) alters GR and MR expression in the developing limbic system in both a region- and sex-specific manner (Dean *et al.*, 2001). The dose (1 mg/kg) used in the guinea pigs is comparable with the dose used in humans (approximately 0.25 mg/kg) since the guinea pig GR has a fourfold lower a nity for dexamethasone than the human GR (Dean *et al.*, 2001). Fetal plasma cortisol concentrations 24 hours after the second injection of dexamethasone were increased in females but decreased in males. This would suggest that the females rebound from the synthetic glucocorticoid-mediated inhibition of HPA function faster than males. The dexamethasone-exposed female fetuses exhibited significantly increased levels of MR and GR mRNA in the hippocampus and dentate gyrus (Dean *et al.*, 2001). These results demonstrate that a single course of synthetic glucocorticoids during the brain growth spurt (Dobbing & Sands, 1979) have profound and sex-specific effects on the fetal HPA axis.

Administration of multiple courses of synthetic glucocorticoids to pregnant guinea pigs affected fetal weight, HPA axis activity, and GR and MR levels, again in a sex-specific manner. Repeated courses of dexamethasone or betamethasone (1 mg/kg; 3 × 2 injections 24 hours apart) on gd 40/41, 50/51, and 60/61, which

represent periods of rapid neurogenesis, brain growth, and myelination, respectively (Dobbing & Sands, 1979), decreased body weight in male, but not in female fetuses (McCabe *et al.*, 2001). Male fetuses whose mothers were treated with either betamethasone or dexamethasone exhibited significantly decreased levels of CRH mRNA in the hypothalamic PVN, but only dexamethasone-exposed males demonstrated significantly increased hippocampal MR and GR mRNA (McCabe *et al.*, 2001). Female fetuses whose mothers were treated with betamethasone demonstrated lower levels of CRH mRNA, but no effect on brain GR and MR levels. In the adult, the blood–brain barrier, through expression of P-gp, can exclude peripherally administered synthetic glucocorticoids from entering the brain (Petropoulos *et al.*, 2007). However, the reduction in CRH mRNA identified in the fetal PVN following maternal treatment with synthetic glucocorticoids provides conclusive evidence that synthetic glucocorticoids enter the fetal brain and inhibit central drive to the fetal HPA axis. It is also noteworthy that repeat courses of high dose synthetic glucocorticoids do not lead to autoregulation of the GR in the fetal brain (McCabe *et al.*, 2001). Therefore, the fetus is unable to protect brain structures that are sensitive to glucocorticoid actions. This latter observation certainly has ramifications for the use of antenatal glucocorticoid treatment in humans.

During fetal development, 5-hydroxytryptamine (5-HT) in uences transcription of hippocampal GR mRNA via the 5-HT_7 receptor. In vitro exposure of fetal hippocampal neurons, derived at gd 50, to 5-HT results in upregulation of GR mRNA, but not MR mRNA (Erdeljan *et al.*, 2005). Previous elegant studies in the rat have shown that the effects of 5-HT on GR are mediated by the transcription factor inducible nerve growth factor A (NGFI-A) (Meaney *et al.*, 2000). Interestingly, there is a rapid elevation in NGFI-A expression (>800%) in the fetal guinea pig hippocampus approaching term and this increase correlates closely with the increase in fetal plasma cortisol concentrations. Further, fetal exposure to dexamethasone can prematurely increase hippocampal NGFI-A expression, suggesting that the late gestation rise in endogenous glucocorticoids is involved in activation of transcription in the fetal hippocampus (Andrews *et al.*, 2004). The functional significance of this activation process remains to be determined, though it likely underlies the increase in hippocampal GR that occurs in late gestation in the guinea pig (Owen & Matthews, 2003).

Relatively few studies of the acute effects of synthetic glucocorticoids on fetal neurodevelopment have been undertaken in other rodents due to their small size and neuroanatomical immaturity. However, in mice, a single injection of dexamethasone (0.4 mg/kg) on gd 15.5 (term ~19.5 days) reduced MR mRNA expression in the hippocampus two hours after dexamethasone administration, but this effect was not observed from gd 18 onwards, indicating a transient effect of synthetic glucocorticoid exposure on the fetal hippocampus (Noorlander *et al.*, 2006). This group also found that GR mRNA was not present in the mouse hippocampus until postnatal day 5 (Noorlander *et al.*, 2006). In rats, administration of dexamethasone (three times daily) to pregnant rats from gd 16 (term ~ 21 days) onward resulted in fetuses that on gd 19 and 21 exhibited decreased ACTH immunoreactivity in the pituitary gland as well as reduced proliferative activity of adrenocortical cells (Stojanoski *et al.*, 2006), consistent with the negative feedback role of glucocorticoids on HPA axis function.

The acute impact of maternal synthetic glucocorticoid treatment on the fetus has been studied in other long-gestation species. Consistent with studies performed in guinea pigs, fetal growth and HPA axis regulation is affected by fetal synthetic glucocorticoid exposure. In the sheep, repeated maternal injections with betamethasone (0.5 mg/kg) on gd 104, 111, and 118 (term ~150 days) resulted in fetal sheep that on gd 146 exhibited significantly higher plasma ACTH levels and a trend toward increased plasma cord cortisol levels (Sloboda *et al.*, 2000). Molecular analysis revealed significantly higher GR mRNA levels in the anterior pituitary, but no difference in CRH of GR mRNA in the hypothalami of fetuses that had been exposed to synthetic glucocorticoids 28 days earlier. While increased pituitary-adrenocortical activity on a background of elevated pituitary GR mRNA might appear counterintuitive, it is likely that there is an overcompensatory rebound of pituitary-adrenal activity in the fetus following earlier exposure to synthetic glucocorticoids. Also, GR protein and glucocorticoid binding was not assessed in the anterior pituitary, and it is possible that although there was an increase in GR mRNA this may not have corresponded to an increase in functional GR in the anterior pituitary. Unfortunately there is no information concerning potential sex differences in the acute response to synthetic glucocorticoid exposure in fetal sheep.

Prenatal glucocorticoid and HPA function after birth

Very little is currently known about the impact of antenatal synthetic glucocorticoid exposure on HPA function in human infants, children, or adults. Follow-up in the original prenatal betamethasone trial (single course therapy) indicated that there was no clinical effect on cardiovascular risk factors at 30 years of age, though there were indications of insulin resistance, particularly in women (Dalziel *et al.*, 2005). More recently, it has been shown that infants whose mothers were treated with betamethasone (12 mg; 1 × 2 injections, 12 hours apart) exhibited a blunted heart rate and salivary cortisol response to a heelstick stressor at both one and 34 weeks postnatally (Davis *et al.*, 2006). This finding of an effect on stress reactivity in infants as a result of antenatal synthetic glucocorticoid exposure warrants further study of long-term effects in a larger population. To date, no studies have assessed HPA function in school-age children or young adults who were exposed to glucocorticoids during fetal life.

Limited studies have been undertaken to establish the long-term effects of fetal exposure to synthetic glucocorticoids on HPA axis function in the nonhuman primate. Pregnant rhesus monkeys treated daily with dexamethasone (4 × 1.25 mg/kg) beginning on 132 days of gestation resulted in 10-month-old offspring that exhibited elevated basal and stress-stimulated cortisol levels (Uno *et al.*, 1994). Maternal oral administration of dexamethasone (0.12 or 0.2 mg/kg) from mid-gestation to term in the Vervet monkey resulted in offspring that at 12 months of age exhibited an elevated cortisol response to stress (de Vries *et al.*, 2007). In the common Marmoset, repeated administration of dexamethasone (5 mg/kg) to pregnant females during either early or late gestation led to significantly lower maternal plasma cortisol levels, but no effect on basal HPA axis activity in offspring from postnatal day 2 to 84 (Hauser *et al.*, 2007).

A large number of studies have been undertaken in the guinea pig. Juvenile male, but not female, offspring that had been exposed to repeated courses of synthetic glucocorticoids in late gestation exhibited a blunted HPA axis response to the stress of maternal separation, but no difference in basal plasma cortisol concentrations (Owen & Matthews, 2007a). Juvenile males euthanized under basal conditions exhibited increased pituitary POMC mRNA and CRH receptor mRNA; and decreased adrenal CYP17 mRNA, an enzyme involved in adrenal steroidogenesis (Owen & Matthews, 2007a). Since molecular analysis was undertaken in animals that were euthanized in a basal state, it was not possible to directly determine the mechanisms associated with the blunted HPA axis response to maternal separation identified following antenatal exposure to synthetic glucocorticoids.

Repeated maternal synthetic glucocorticoid treatment (three courses; gd 40/41, 50/51, 60/61) results in a substantial reduction in basal plasma ACTH and cortisol secretion in adult male offspring (Liu *et al.*, 2001). There was a decrease in hypothalamic CRH mRNA expression indicating reduced central drive to the pituitary-adrenal axis in these animals. Furthermore, there was increased hippocampal MR mRNA expression, suggesting increased glucocorticoid-negative feedback at the level of the hippocampus, and a resultant feed-forward inhibition of CRH in the PVN. Interestingly, basal plasma testosterone concentrations were significantly increased in these males (Liu *et al.*, 2001). Testosterone inhibits HPA function (Viau, 2002), thus the reduction in HPA axis function in adult male guinea pigs following synthetic glucocorticoid exposure is likely modulated, at least partially, by increased testosterone concentrations.

In adult female offspring, repeated antenatal synthetic glucocorticoid treatment also resulted in reduced pituitary–adrenal activity when animals were in the luteal (progesterone-predominant) phase of the reproductive cycle, resembling the situation in males (Liu *et al.*, 2001). Intriguingly, the same animals exhibited increased pituitary–adrenal activity at estrous, indicating interaction of the reproductive cycle with the programming effects of antenatal synthetic glucocorticoid exposure. In longitudinal studies, using salivary cortisol measurements, we have shown similar results in adult female offspring at 150 days of age (Dunn *et al.*, 2005). In summary, HPA function in guinea pigs is consistently suppressed in male and female offspring following repeated antenatal treatment with synthetic glucocorticoids, leading to hypocortisolemia. In females, there is a significant interaction with reproductive cycle.

As in the guinea pig, there are long-term effects of prenatal synthetic glucocorticoid exposure on HPA axis function and regulation in the rat. Maternal dexamethasone (0.4 mg, i.p.) treatment on gd 17 and 19

(term ~22 days), had no effect on basal HPA function in prepubertal offspring, though differences in the ratio of AVP and CRH protein content in the median eminence of these young rats were observed (Bakker *et al.*, 1995). In another study, daily maternal treatment with dexamethasone (0.1 mg/kg) during the third week of gestation resulted in neonatal rat offspring of both sexes that exhibited decreased HPA axis function associated with decreased CRH mRNA in the hypothalamic PVN (Burlet *et al.*, 2005). However, adult males exposed to the same prenatal dexamethasone regimen exhibited significantly increased basal corticosterone levels and this was associated with lower hippocampal MR and GR mRNA expression (Levitt *et al.*, 1996). In a more recent comprehensive study from the same group, maternal dexamethasone treatment in the last week of gestation significantly increased basal ACTH and corticosterone levels in male but not female offspring. However, the changes were specific to the time of day; HPA activity was increased in the morning (0800 hours), but not at the diurnal peak (2000 hours) (O'Regan *et al.*, 2004).

More recently, it has been shown that daily maternal treatment with a higher dose of dexamethasone (0.125 mg/kg) during the last week of gestation resulted in adult male rats that exhibited a prolonged corticosterone response to restraint stress. This was associated with increased CRH mRNA in the hypothalamus, decreased MR mRNA and increased 11β-HSD1 mRNA in the hippocampus (Shoener *et al.*, 2006). Hippocampal 11β-HSD1 converts inactive glucocorticoid to active cortisol or corticosterone, thereby increasing the local availability of biologically active glucocorticoids. Indeed, another study using a similar prenatal dexamethasone regimen also found increased 11β-HSD1 in neonatal hippocampal cells (Wan *et al.*, 2005), suggesting that dexamethasone upregulates this enzyme which in turn may lead to an increased hippocampal exposure to endogenous glucocorticoid.

A recent series of studies has shown sex differences in pituitary regulation following antenatal dexamethasone exposure (John *et al.*, 2006). Adult males displayed a reduction in pituitary corticotroph number and impaired granule margination, an aspect of corticotroph morphology, following antenatal synthetic glucocorticoid exposure (Theogaraj *et al.*, 2005). In contrast, adult females exhibited increased pituitary corticotroph granule density and margination (John *et al.*, 2006), indicating sex-specific effects of antenatal synthetic glucocorticoid exposure on HPA regulation in adult life.

Again, a number of studies have been undertaken in other species that give birth to neuroanatomically mature young. Longitudinal analysis in sheep identified that antenatal synthetic glucocorticoid exposure is associated with alterations in HPA function that vary as a function of age. A single maternal injection of betamethasone (0.5 mg/kg) on gd 104 had little effect on HPA function in offspring at 6 months of age but led to offspring with significantly elevated basal and stimulated plasma cortisol concentrations at 1 year of age and significantly lower brain weight at 3.5 years. At 2 years of age, offspring whose mothers had been repeatedly treated with betamethasone during pregnancy exhibited elevated ACTH responses to a CRH/AVP challenge. These animals also exhibited increased basal ACTH levels but reduced basal and stimulated cortisol levels (Sloboda *et al.*, 2007). The fact that the effects of antenatal synthetic glucocorticoid exposure change with advancing age indicates the importance of studying outcomes at several different times during the life course.

Although a number of sophisticated studies in rats, sheep, and guinea pigs have shown long-term profound effects on HPA function, care must be applied in extrapolation to the human due to variability in neurodevelopmental profiles. Collectively, animal studies show that short-term exposure to synthetic glucocorticoids during vulnerable windows of brain development permanently affects HPA axis function in offspring. Increased HPA tone throughout life will have a considerable impact on adult health, due to elevated tissue exposure to glucocorticoids (Fig. 22.3). In humans, elevated levels of cortisol have been associated with increased atherosclerosis, cholesterol levels, incidence of diabetes, immunosuppression, and cognitive impairment. Animal studies have found similar effects of chronically elevated HPA activity (Lupien & McEwen, 1997). Taken together, these data would indicate that chronically elevated HPA function leads to accelerated aging. In contrast, lifelong exposure to reduced HPA activity may tend to slow down the onset of age-related pathologies. However, reduced HPA axis activity has been linked to a number of neuropathologies in humans including posttraumatic stress disorder (Fries *et al.*, 2005)

Prenatal glucocorticoids and behavior

Follow-up studies for the early human trials that involved fetal exposure to a single course of synthetic glucocorticoid, failed to identify an effect of prenatal betamethasone on cognitive function, working memory,

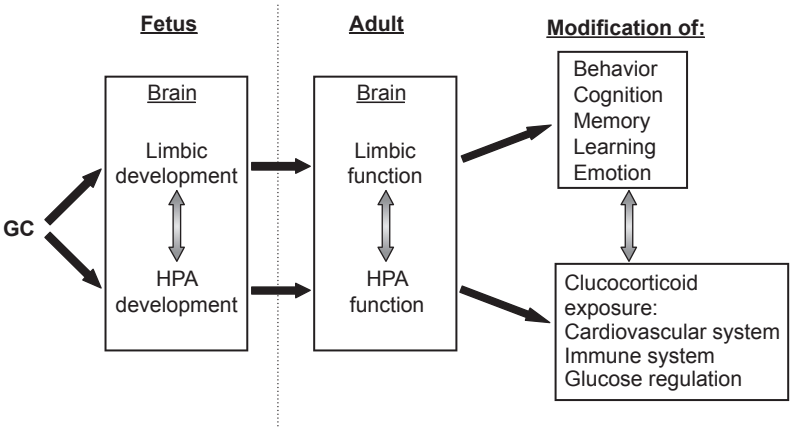

Fig. 22.3 Diagrammatic representation of the routes by which prenatal glucocorticoid (GC) exposure programs adult behavior and neuroendocrine function. The fetal limbic system (primarily the hippocampus), hypothalamus, and anterior pituitary express high levels of corticosteroid receptors, and are sensitive to glucocorticoids. Exposure to exogenous glucocorticoid at this time will alter development and subsequent function of both the limbic system and the hypothalamo–pituitary–adrenal (HPA) axis. The hippocampus regulates HPA function, and endogenous glucocorticoids (the end product of HPA activation) modify many aspects of limbic function. The gray arrows indicate this close functional association. In the periphery, the overall effect of programming during development will be altered exposure to endogenous glucocorticoids throughout life. Increased exposure will predispose to a number of neurological, metabolic, and cardiovascular diseases, whereas reduced exposure may act to protect against these pathologies. See text for further details.

psychiatric morbidity, handedness, or health-related quality of life (Dalziel *et al.*, 2005). However, in a retrospective study, increasing courses of betamethasone in utero led to an increased risk of aggressive behavior in children at both 3 and 6 years of age. These children also exhibited increased distractibility and hyperkinetic behavior (French *et al.*, 2004). These latter studies are consistent with those undertaken in animal models, and indicate that antenatal exposure to synthetic glucocorticoids leads to symptoms in children analogous to attention-deficit hyperactivity disorder (ADHD).

In rhesus monkeys, there was no effect of prenatal dexamethasone treatment (1.25 mg/kg/day for four days) at approximately 75% of gestation on physical development or locomotor behavior in young primates, although there were major differences in hippocampal structure (Uno *et al.*, 1994) (see below). In the common Marmoset, repeated dexamethasone (5 mg/kg) administered to pregnant females during the early gestation period resulted in infants that exhibited increased bodyweight. This was associated with increased time spent eating solid food and an increase in mobility. These infants also exhibited an increase in solitary play and a greater amount of tail hair piloerection, indicating activation of the sympathetic nervous system (Hauser *et al.*, 2007).

Multiple courses of betamethasone (1 mg/kg; 3 × 2 injections 24 hours apart on gd 40/41, 50/51, and 60/61) administered to pregnant guinea pigs resulted in female juvenile offspring (postnatal day 10) that exhibited significantly increased locomotor activity in an open field (Owen & Matthews, 2007b). There was no effect of antenatal synthetic glucocorticoid exposure on locomotor activity in juvenile males, again indicating sex-specific effects. In humans and in animal models, hyperactivity has been linked to dopamine signaling in the nucleus accumbens (NAcc) and the medial prefrontal cortex (mPFC) (Meaney *et al.*, 2002). In this regard, hyperactive rats show increased dopamine levels and a reduction in dopamine transporter (DAT) activity (Meaney *et al.*, 2002). This would suggest that the hyperactivity identified in female offspring following antenatal exposure to repeated betamethasone may result from glucocorticoid actions on the developing dopamine signaling system, and further work is required to determine whether this is indeed the case.

We have demonstrated that antenatal exposure to synthetic glucocorticoids leads to acute downregulation of mRNA expression of the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) receptor in the hippocampus of the female fetus (Owen & Matthews, 2007b). This appears to be a long-term effect, as similar downregulation of the hippocampal NR1 subunit occurred in juvenile female offspring (postnatal day 10) that were repeatedly exposed to synthetic glucocorticoids during fetal life (Owen & Matthews, 2007b). There was no difference in any of the NMDA receptor

subunits in male fetuses or juvenile males that were exposed to repeated treatments with synthetic glucocorticoids, again indicating sex specificity of effects.

The NMDA receptor has a well-established role in long-term potentiation (LTP), an example of neuronal plasticity, which is thought to underlie learning and memory formation (Setiawan *et al.*, 2007). Given the changes that we identified in the NMDA receptor system in juvenile offspring following antenatal exposure to synthetic glucocorticoids, it would be anticipated that there would be alterations in LTP and learning and memory. Interestingly, in vitro analysis of LTP in hippocampal slices derived from young guinea pigs that had been exposed repeatedly to synthetic glucocorticoids in utero, revealed no effect of antenatal synthetic glucocorticoid exposure on LTP under basal conditions (Setiawan *et al.*, 2007).

Daily maternal treatment with dexamethasone over the last week of gestation resulted in adult male rats that exhibited reduced exploration in an open field and elevated plus maze behavioral hypoactivity (Welberg *et al.*, 2001). These males also exhibited increased CRH mRNA in the central nucleus of the amygdala and hypothalamic PVN as well as reduced GR and MR mRNA expression in the hippocampus (Welberg *et al.*, 2001). Amygdaloid CRH plays an important role in fear-related behaviors and the increase in CRH mRNA in this region may be responsible for increased fear and a corresponding decrease in exploration in these animals. In contrast to the synthetic glucocorticoid regimen used by Welberg and colleagues, maternal dexamethasone (0.05 mg/kg) treatment on gd 17, 18, and 19 resulted in adult male offspring that exhibited significantly increased activity and rearing in an open field and a greater corticosterone response to exposure to the open field (Muneoka *et al.*, 1997). In another study, repeated treatment with synthetic glucocorticoids resulted in young offspring that performed poorly on motor function tests during infancy (Burlet *et al.*, 2005). Together, these studies highlight the importance of dosage and timing of exposure on the behavioral phenotype observed.

Glucocorticoids and central neurotransmitter systems

The catecholamines, epinephrine and norepinephrine, stimulate HPA function via α_1-epinephrinergic receptors (Herman *et al.*, 1996). Catecholaminergic innervation of the parvocellular PVN is derived principally from the caudal medulla. Brainstem catecholaminergic systems are also implicated in hippocampal function. Stress increases norepinephrine turnover in the hippocampus and PFC (Herman *et al.*, 1996), and norepinephrine has been shown to modulate hippocampal corticosteroid receptor activity.

Dexamethasone exposure in the last week of gestation results in adult offspring with reduced norepinephrine turnover in the cerebellum and forebrain (Slotkin *et al.*, 1992) and reduced norepinephrine content in the hippocampus and neocortex (Muneoka *et al.*, 1997). However, longitudinal studies have shown that antenatal glucocorticoid exposure results in premature maturation of norepinephrine systems in the brainstem, forebrain, and cerebellum (Slotkin *et al.*, 1992), and induces over-expression of the norepinephrine transporter. Unfortunately, norepinephrine levels and turnover, and norepinephrine transporter levels have not been measured in the hypothalami of offspring that were exposed to glucocorticoids as fetuses. No effect of prenatal dexamethasone on epinephrinergic receptors has been detected (Slotkin *et al.*, 1992). Without further measurements in the hypothalamus it is unclear how dexamethasone-induced modification of adult brain norepinephrine systems relates to the increase in HPA function. However, it has been shown that alterations in hippocampal norepinephrine can induce changes in corticosteroid receptor levels, and therefore modify glucocorticoid-negative feedback. Antenatal glucocorticoids have also been shown to promote early maturation of dopamine systems in the forebrain (Slotkin *et al.*, 1992), and this is likely associated with changes in behaviors noted in these animals.

Ascending serotonergic neurons project directly to the parvocellular PVN and increase activity of CRH-containing neurons, stimulating CRH and ACTH secretion into the hypophyseal portal blood (Herman *et al.*, 1996). There is also a rich serotonergic innervation of the hippocampus (Muneoka *et al.*, 1997). Prenatal exposure to dexamethasone in the last week of gestation leads to male offspring with increased hypothalamic and medullary serotonin (5-HT) levels, but reduced hippocampal 5-HT turnover (Muneoka *et al.*, 1997). Prenatal glucocorticoid exposure also promoted brainstem 5-HT transporter development, and resulted in increased transporter activity throughout the life of the animal (Slotkin *et al.*, 1996). Measurement of transporter activity was only undertaken in the brainstem, and potential glucocorticoid-induced changes in

the hippocampus and hypothalamus remain to be determined. Alterations in 5-HT transporter function and increased hypothalamic 5-HT levels are likely responsible, in part, for the elevation of HPA activity observed in adult offspring. The effect of 5-HT on HPA function is not confined to the PVN as 5-HT tonically inhibits the expression of hippocampal MR in adult male rats (Semont *et al.*, 1999).

Prenatal glucocorticoid and brain structure

Several studies in young animals and adults have demonstrated that stress and increased glucocorticoid can have a major impact on hippocampal structure (de Kloet *et al.*, 2005). Perhaps the most striking of these are the observations that stressful experience or exposure to chronically elevated glucocorticoids results in significant reductions in hippocampal volume (Bremner *et al.*, 1995; Sapolsky, 1996; Stein *et al.*, 1997).

An elegant series of studies carried out in the rhesus monkey have considered the impact of short periods of fetal glucocorticoid exposure during late gestation on structural development of the limbic system (Uno *et al.*, 1990, 1994). The effect of single injections of dexamethasone (0.5, 5, and 10 mg/kg) at 132 days of gestation (term ~185 days) was assessed on gd 135. There was considerable dose-dependent neuronal degeneration in the CA1–CA3 hippocampal pyramidal neurons, shrinkage and condensation of neuron soma and dendritic branches, depletion of the number of neurons, and disintegration of mossy fiber endings in the zona lucidum (Uno *et al.*, 1990). Fetuses receiving multiple injections of lower doses (0.125 × 4, 1.25 × 4, and 2.5 mg/kg × 4) showed more severe damage than those receiving a single larger injection. This would be more analogous to the treatment regimen in pregnant women, and the lower dose would be similar to that prescribed for suspected preterm labor (NIH Consensus Panel, 1995).

Another group of fetuses was studied 30 days after exposure, and although the acute neurodegenerative changes seen in the brains of the day 135 fetuses had subsided, the size of the perkaryonic soma of the pyramidal neurons was small and their dendritic branches with the mossy fiber endings in the CA3 regions were poorly developed compared with normal age-matched controls (Uno *et al.*, 1990). In the dentate gyrus, the density of the granular neurons was less and the individual sizes of the neurons were smaller. Overall, the number of hippocampal neurons at day 162 was approximately 30% reduced in the dexamethasone-treated fetuses, and the size of the whole hippocampal formation was reduced (Uno *et al.*, 1990). In a further experiment involving dexamethasone-treated fetal monkeys, the hippocampal volume was analyzed at 20 months of age by magnetic resonance imaging (MRI). This revealed an approximate 30% reduction in hippocampal volume in offspring exposed to dexamethasone (4 × 1.25 mg/kg) at day 132 of gestation (Uno *et al.*, 1994). Maternal stress in monkeys, which increased maternal plasma cortisol concentrations, for six weeks resulted in an equivalent reduction in hippocampal size comparable with the dexamethasone effects and which were evident at 3 years of age (Coe *et al.*, 2003).

Human and animal studies have demonstrated that morphological changes in the hippocampus are associated with a number of functional consequences (Sapolsky, 1996). Bremner *et al.* (1995) showed that male combat veterans with posttraumatic stress disorder had reduced MRI-derived right-sided hippocampal volume compared with control subjects, and that certain aspects of their memory deficit were correlated with hippocampal volume. Other studies have demonstrated a reduction in hippocampal volume in women victimized by childhood sexual abuse, and that the severity of psychiatric complications was correlated to reduction in hippocampal volume (Stein *et al.*, 1997). It is therefore likely that glucocorticoid-induced changes in hippocampal structure during development have long-term behavioral and neuroendocrine consequences.

The mechanisms by which glucocorticoid-induced damage to the hippocampus takes place are not well understood. It is generally accepted that glucocorticoids promote differentiation over proliferation (de Kloet *et al.*, 2005). Animal studies have demonstrated that glucocorticoids interfere with normal rates of cell birth and death that occur during development. However, it is also known that glucocorticoid exposure can indirectly damage mature neurons (Sapolsky, 1996). It has been shown that glucocorticoids block the uptake of glucose into neurons. Under resting conditions, glucocorticoids do not reduce energy metabolism su ciently to kill neurons, however, during situations such as ischemia or glucose insu - ciency, glucocorticoids decrease energy metabolism, making neurons more vulnerable to challenge. It is thought that glutamate, which is released during episodes of hypoxia and hypoglycemia is responsible for inducing damage in compromised neurons (Sapolsky,

1996). Glucocorticoids have also been shown to increase extracellular glutamate levels in the hippocampus, by preventing glutamate reuptake, and therefore further exacerbating damage. This damage is consistent with that described in fetal primates exposed to glucocorticoids.

It is possible that compromise (i.e., reduction of pyramidal neurons or alterations in axonal/dendritic processes and synaptogenesis) in the developing hippocampus may decrease the age at which hippocampal deficits are first noted. Prenatally programmed increases in HPA function, may exacerbate this hippocampal deficit (Sapolsky, 1996), and in turn will lead to further increases in HPA function (due to reduced glucocorticoid negative feedback). In this respect, aging humans tend to undergo a decrease in HPA resiliency (ability to switch off the HPA response), and it has been suggested that this deficit is linked to lifelong exposure to glucocorticoids. For these reasons, follow-up studies in humans and animal models, to investigate the impact of prenatal glucocorticoid exposure, may fail to observe significant hippocampal deficits until adulthood or early old age.

Transgenerational effects of synthetic glucocorticoids

Recent studies have begun to investigate the possibility that the effects of antenatal synthetic glucocorticoid exposure are manifest across multiple generations. Transgenerational effects of synthetic glucocorticoids have been identified in the rat. Female F_1 offspring whose mothers were treated with dexamethasone (0.1 mg/kg) in the last week of pregnancy were mated with males from the same prenatal treatment. Both the F_1 and F_2 generation offspring exhibited decreased birthweight compared with controls. Hepatic phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in gluconeogenesis, was significantly increased in F_2 males despite no manipulation of the F_1 pregnancy. Consistent with this finding, glucose was significantly elevated in F_2 males in response to an oral glucose tolerance test (Drake *et al.*, 2005). Clearly, further studies are required to determine the mechanisms of transgenerational programming following antenatal synthetic glucocorticoid exposure. Mechanistically, there are likely two primary routes by which the effects of antenatal synthetic glucocorticoids pass to the next generation. The first involves altered maternal endocrine and cardiovascular adaptation to pregnancy in the F_1 offspring. This would in turn lead to altered endocrine regulation in offspring. The second involves epigenetic mechanisms.

Potential mechanisms of programming: epigenetic modification

There are a number of routes by which antenatal glucocorticoid exposure might permanently affect HPA function and behaviors in F_1 offspring. These might include structural effects during development that lead to permanent changes in the wiring of the brain and therefore its function. Alternatively, there may be permanent effects on the expression of genes important in regulation of HPA function and behavior. Permanent changes in gene expression following fetal exposure to glucocorticoid are likely to involve epigenetic modification. Chromatin and DNA modifications are stable over rounds of cell division and do not involve changes in the underlying DNA sequence. Epigenetic changes play a role in the process of cellular differentiation, allowing cells to maintain different characteristics despite containing the same genomic material (Weaver *et al.*, 2007). Examples of epigenetic modifications include changes in the methylation of cytidine-guanosine (CpG) nucleotides in the regulatory regions of specific genes and changes in chromatin structure through histone acetylation and methylation. Epigenetic modification of histones or of DNA itself modulates the rate of transcription to mRNA by controlling access of transcription factors to the DNA sequence. In this regard, CpG sequences in the promoter regions of actively transcribed genes are generally unmethylated, which allows for binding of transcription factors. Transcriptionally inactive chromatin is characterized by histone deacetylation, promoter CpG methylation, and subsequent decreased binding of transcription factors (Szyf, 2007).

In the rat, a restricted protein diet during pregnancy resulted in decreased methylation of the hepatic peroxisome proliferator-activated receptor α (PPARα) and GR promoters in young rat offspring. The decreased methylation was associated with increased expression of PPARα and GR mRNA. Interestingly, the hypomethylation was prevented by supplementation of the restricted protein diet with folic acid, suggesting that the change in methylation status may be due to impaired folic acid transfer from mother to fetus (Lillycrop *et al.*, 2005). In order to determine whether

the altered methylation status was passed on to subsequent generations, adult female rats (F_1) – whose mothers had been exposed to a low protein diet during pregnancy – were mated, and hepatic gene promoter expression was determined in their offspring at 80 days of age. Methylation of hepatic PPARα and GR promoters was significantly lower in the F_1 and F_2 generations, and there was a trend toward higher expression of PPARα, GR, acyl-CoA oxidase, and significantly higher PEPCK expression, despite no dietary manipulation of the pregnant F_1 offspring (Burdge *et al.*, 2007).

Elegant studies in the rat have shown that the early postnatal environment can also permanently modify epigenetic marks in the genome. In rat dams, maternal behaviours such as licking and grooming (LG) and arched back nursing (ABN) have naturally occurring individual differences. Adult rats of mothers that exhibited a high level of maternal care during the first ten days of their life were shown to demonstrate significantly lower plasma ACTH and corticosterone responses to stress, increased hippocampal GR mRNA and decreased CRH mRNA (Liu *et al.*, 1997). Behaviorally, these offspring also exhibited decreased behavioral fearfulness in response to novelty compared with offspring from mothers that exhibited low levels of maternal care (Caldji *et al.*, 1998). The endocrine and behavioral phenotype observed in these animals was not due to genetic inheritance, but rather due to differences in the level of maternal care, as cross-fostering experiments showed that offspring of low-care mothers reared by high-care mothers were also less fearful in a novel environment (Francis *et al.*, 1999). Further molecular analysis revealed that epigenetic modification of the hippocampal GR promoter was, at least in part, responsible for these endocrine and behavioral changes. It was discovered that expression of the exon 1_7 of the GR promoter was increased in the offspring of high-care mothers. Further analysis revealed a decrease in methylation within exon 1_7 of the GR promoter in the adult offspring of high-care mothers. The site of reduced methylation corresponded to a binding site for the transcription factor, NGFI-A, and was associated with significantly greater histone acetylation and subsequently greater binding of NGFI-A protein to hippocampal exon 1_7 GR promoter in adult offspring of high-care mothers (Weaver *et al.*, 2004). Developmental analysis revealed that this methylation pattern emerged in offspring between one and six days of postnatal life.

To further establish that the increased methylation status of the offspring of low-care mothers was responsible for the decreased expression of hippocampal GR, a histone deacetylase inhibitor, trichostatin A (TSA), was administered. TSA treatment significantly decreased the degree of cytosine methylation within the NGFI-A binding region of the exon 1_7 GR promoter in the offspring of low-care mothers compared with vehicle controls. This led to increased GR expression in the hippocampus and a decreased corticosterone response to stress which resembled that of offspring of high-care mothers (Weaver *et al.*, 2004).

This series of studies has demonstrated epigenetic modification of genes relevant to HPA axis activity and behavior are adapted in response to the early environment. They have also highlighted the potential for epigenetic modification as a mechanism by which maternal antenatal synthetic glucocorticoid exposure can lead to permanent changes in the physiology of the fetus. Indeed, there is now some evidence that glucocorticoid can modify the methionine cycle and affects promoter methylation. Clearly, further work is required in this rapidly emerging field.

Conclusion

There are long-term effects of antenatal synthetic glucocorticoid exposure on HPA axis activity in all species studied. It appears that synthetic glucocorticoids are able to affect fetal HPA axis development causing permanent changes in the HPA axis that persist throughout life. The consistent finding of sex differences in the endocrine phenotype following antenatal synthetic glucocorticoid exposure provides evidence that programming of the HPA axis involves significant interactions with other systems including HPG function. Further studies are required to delineate these changes as they could potentially affect reproductive function in synthetic glucocorticoid-exposed offspring. Profound behavioral changes have also been identified in animals exposed to synthetic glucocorticoid in utero. Locomotor activity is particularly affected suggesting synthetic glucocorticoids act to program the dopamine signaling systems. In line with the animal data, a retrospective human follow-up study has demonstrated ADHD-like symptoms in children exposed to repeated courses of synthetic glucocorticoids in utero. Since similar central signaling systems are involved in both the regulation of activity and attention, future studies need to investigate attention systems in animal models following antenatal synthetic glucocorticoid exposure.

In conclusion, both single and multiple courses of synthetic glucocorticoids have acute effects on the

fetus and cause long-term changes in endocrine function and behavior during postnatal life in the F_1 generation. Intriguingly, animal studies have identified profound effects in subsequent generations indicating transgenerational transmission. Mechanistically, synthetic glucocorticoids likely impart their effects by modification of the epigenome in the early environment. Further studies are required to fully investigate this possibility as epigenetic modifications offer potential for reversal of the effects of synthetic glucocorticoids. This knowledge will allow the development of improved therapeutic strategies for management of preterm delivery, which affects almost 10% of all pregnant women.

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Section 6
Chapter
23

Consciousness

On the emergence of consciousness

Hugo Lagercrantz and Jean-Pierre Changeux

Introduction

One of the most important challenges of this century is to understand the neural bases of consciousness from the molecular to the highest cognitive level, in other words, to bridge "the hump from neurochemistry to subjective feeling" (Searle, 2000). Yet, due to the complexity of the human brain, it is not anticipated that a single concept, method, or experimental finding will ever give the decisive answer. On the other hand, studying the brain from early fetal stages in utero up to the adult state may offer fruitful insights to understand human consciousness. The approach is in itself rather new. It is, indeed, quite surprising to note that William James regarded the infant as unaware of its body, of itself (the I) and of the outside world, i.e., "the blooming buzzing confusion." Sigmund Freud also claimed that children up to 5 years of age are rarely aware of the motives and reasons for their behavior and that the bulk of their mental life is unconscious. Lev Vygotsky was the first psychologist in modern time who thought that a baby's view of the world was nearly as complex and highly structured as the adult one (see Gopnik *et al.*, 1999; Zelazo, 2004). Also, at variance with the commonly accepted view that consciousness corresponds to a unique and irreducible system, developmental data argue for a progressive, stepwise, growth of the diverse components of adult consciousness (Zelazo, 2004).

This chapter is deliberately limited to the newborn brain, aware of the fact that the infant subsequently has access to higher levels of consciousness such as, after 15–18 months, "self-consciousness," together with working and episodic memory, language, and self-recognition in mirror tests.

Definitions of consciousness

A first difficulty is to agree on the definition of what it actually means to be conscious in scientific terms. For Searle (2000), "Consciousness consists of inner, qualitative, subjective states and processes of sentience or awareness." For Merker (2007) it can be defined "most simply as the 'medium' of any and all possible experience"; "accordingly, to see, to hear, to feel or otherwise to experience something is to be conscious, irrespective of whether in addition one is aware that one is seeing, hearing, and so forth"; "reflective awareness is thus more akin to a luxury of consciousness on the part of certain big brained species." Adopting the point of view of the clinical assessment of patients, one may single out three main components of consciousness: *vigilance*, and, in addition, *mental content* and *selective attention*. Most cognitive scientists further characterize consciousness together with access to one's autobiography and mental time, self-description, and self-agency (Morin, 2001) They stress the importance of being conscious of something, in other words, the ability to give a *report* of the conscious subjective experience (Baars *et al.*, 2003). There must be a global "space or scene," where some kind of *synthesis* between past, present, and future takes place in a continuously changing and dynamic, "flow of consciousness" (Changeux & Dehaene, 2008)

It is essential to distinguish between the "state" of consciousness (e.g., wakefulness, sleep, coma, general anesthesia), the "content" of the conscious experience, and the "level of consciousness."

States of consciousness or vigilance

The global activating signals that determine and control the "states" of consciousness arise from brain stem and diencephalic subcortical structures such as the basal ganglia, ventrolateral thalamus, superior colliculus, median raphe, and the reticular activating system (see Hobson, 1999; Paus, 2000; Boly *et al.*,

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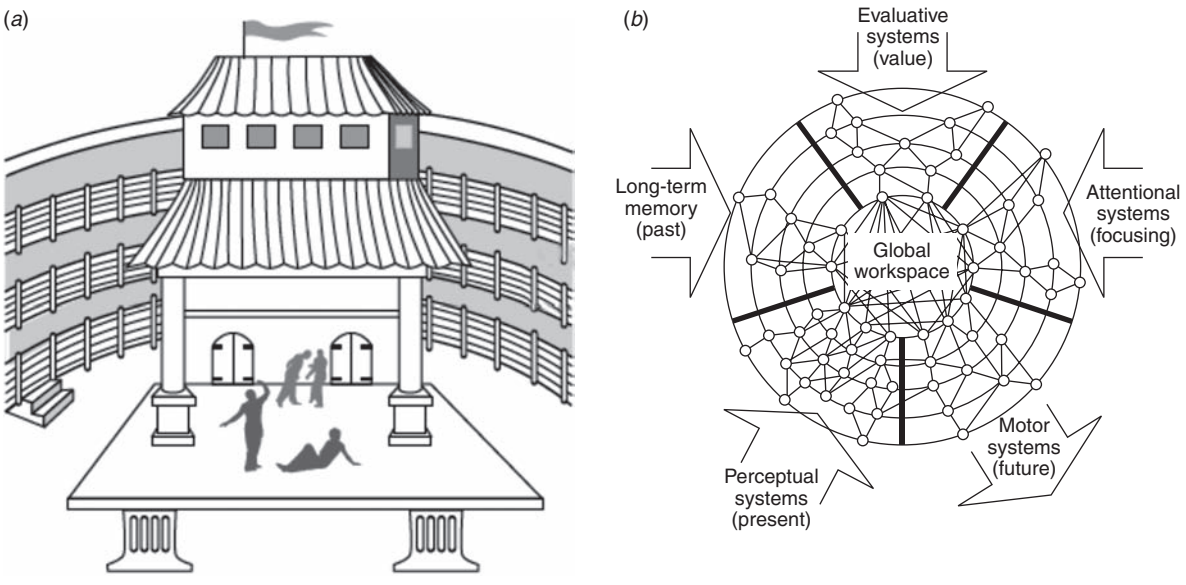

Fig. 23.1 Conscious processing is proposed to be mediated by a "neuronal workspace" made up of reciprocally interconnected neurons with long-range connections that exchange signals with automatic and nonconscious perceptual, attentional, and evaluating processors, which include long-term memories and the self (Dehaene *et al.*, 1998). A metaphor of the global workspace is a theater scene as proposed by Baars (2002). A number of events occur on the stage and other events occur behind the scenes or in the auditorium: conscious processing is assumed to be what is happening on the stage. The prefrontal cortex contributes to the neuronal workspace because of its richness in neurons with long-range axons. It contributes to the selection of what is attended and interprets it to a voluntary action. The application of this model to the newborn is discussed in this chapter.

2008), including cholinergic and monoaminergic neurons arising from the brain stem (see below). Much if not all of the influence exerted by these pathways is mediated by the thalamus and characterized by an increase in the excitability of the cortico-thalamic neurons (Llinas & Steriade, 2006).

Content of consciousness

On the other hand, the telencephalon is viewed as processing the content of consciousness through a horizontal network of pyramidal neurons with long-distance connections (Goldman-Rakic, 1988). They are assumed to contribute to a conscious "global neuronal workspace" (GNW; Fig. 23.1) (Dehaene *et al.*, 1998).

These neurons process information in a *top-down* manner. Their contribution to cognition greatly increases in the course of mammalian evolution together with the expansion of the prefrontal cortex. One way to assess the *content* of newborn consciousness would thus be to study the mobilization of the prefrontal, cingulate, and parietal association areas (Dehaene *et al.*, 2006; Changeux & Dehaene, 2008) where the long axons of the pyramidal neurons are particularly abundant.

Alternative views to the neuronal workspace model have been suggested for the "neuronal correlates of consciousness." For instance, Crick and Koch (2005) suggest a possible role of the claustrum integrating the conscious percepts. Tononi and Edelman (1998) proposed that there are no specialized circuits for access to consciousness but a "dynamic core" consisting of correlated activities of a large number of neurons distributed in the cortex, thalamus, and the limbic system. The relative merits of these diverse hypotheses have been recently discussed (Dehaene & Changeux, 2005).

Levels of consciousness

Consciousness cannot be viewed as an irreducible and unique global entity. The diverse features of the content of adult consciousness do not develop at once, but progressively during fetal and postnatal life as well as in the course of phylogenesis. It thus appears useful to try to cleave the processing of the conscious content in the course of evolution and development into a few "nested levels" of processing (Zelazo, 2004; Changeux, 2006). A lower level of *minimal consciousness* may be assigned to rats or mice, which possess the capacity to create representations,

for instance from visual and auditory experience, to make them accessible to context, store them into long-term memory, to use them for approach and avoidance behavior and to what is referred to as "exploratory behavior" (see Thinus-Blanc *et al.*, 1996; Granon *et al.*, 2003; Koch, 2004). As we shall see, a higher level, referred to as *basic consciousness*, is reached by full-term newborn infants (Changeux & Dehaene, 2008), but it is only at around 15–18 months (therefore far beyond the age limit considered in this chapter), that infants start to exhibit *self-consciousness* (see Zelazo, 2004).

Development of the newborn brain

Neurons: the "elements" of consciousness

Following on from the definition that Robert Boyle gives of the "elements" in his *Sceptical Chemist*, one may think of the neurons as the "elements" of perception, memory, thought, and action, and thus the "elements of consciousness." They differ from other cells, for example from the cells of the skin or the liver, as they have a huge number of connections (an average of 5000 synaptic contacts per neuron) that form the basis of complex networks which make the information from the environment explicit and available for conscious thinking (see Koch, 2004). However, this is not the case for the neurons in the embryo and early fetus, which have not yet grown their axons and dendrites and are thus "naked" without or with only a few synaptic contacts. These connections become progressively established during the development of the human brain, which is a particularly long process compared with that of the brain of a rat or the monkey, i.e., almost half of the lifespan of the early *Homo sapiens* (see Chapter 1).

Milestones of brain development

After formation of the neural tube and the "ballooning" of the brain (Britto *et al.*, 2002), neurons are generated at a speed of about 200 000 new neurons per minute from the tenth until the twentieth gestational week (see Chapter 2). The neurons migrate from the proliferative ventricular zone, traversing the subplate zone, which contains waiting afferents from several sources such as the thalamus and monoaminergic neurons, and settle in the interface between the developing cortical plate and marginal zone to form the cortical layers (Rakic, 1988). There is also a tangential migration of neurons from the ganglion eminence in the telencephalon to the thalamus (Letinic *et al.*, 2002). This second late wave of migrating neurons may contribute to the expansion of the thalamic nuclei that are anatomically related to the association cortex involved in higher cognitive functions and human consciousness.

The next step is the outgrowth of the dendritic and axonal arborizations of the neurons followed by synaptogenesis. There is overproduction of synapses (up to one million per second) during the few months following birth (Chapter 5, Bourgeois, 1997; Huttenlocher, 2002). The exuberant increase of synaptic connections is accompanied by a process of synaptic elimination and stabilization that lasts until adolescence (see Chapter 5). Although myelination of major sensory and motor nerves begins prenatally, this process is not completed in the frontal cortex until the third decade following birth (Sowell *et al.*, 2004; Toga *et al.*, 2006). This may be of importance for the executive functions contributed by the cerebral cortex since myelination results in a nearly 100-fold increase in conduction speed of nerve fibers. Under these conditions, the newborn brain would be in an intermediate "transition" stage of development with an almost adult number of neurons but an immature set of connections (Fig. 23.2).

Fig. 23.2 Immature neurons are naked and have very few connections (*a–e*) compared with more mature ones (*A–D*). (From Ramon y Cajal.)

Neural circuits related to consciousness

The subcortical nuclei and neuronal networks of the "centrencephalic" system that control the states of consciousness (Merker, 2007) develop earlier than the thalamocortical and corticocortical connectivity involved in the content of consciousness. After about 24 weeks, there is an ingrowth of thalamocortical axons in the somatosensory, auditory, visual, and frontal cortices (Kostovic & Jovanov-Milosevic, 2006), and thalamocortical pathways mediating pain perception become functional around the 29–30th week of gestation (Lee *et al.*, 2005). At about this time, a synchrony of the EEG rhythm of the two hemispheres becomes detectable together with the maturation of callosal connections (from 34 weeks), the long-range connections developing long before the elaboration of short-range corticocortical connections (Vanhatalo & Lauronen, 2006). Cortical pyramidal neurons in the primary visual cortex of the human sprout increasingly from the twenty-sixth week (Purpura, 1982) with the development of dendritic spines, which at birth have not reached adult density but suffice for the detection of visual-evoked potentials (VEP) in preterm infants.

A detailed analysis of the development of local circuits, for instance in the human visual cortex (Burkhalter, 1993), reveals that the *vertical* (intracolumnar) connections between layers II/III and V, which link neurons representing the same point in the visual field, develop prenatally at 26–29 weeks of gestation. In contrast, *horizontal* (intercolumnar) connections between different points in the visual field develop later. They first emerge prenatally at about 37 weeks of gestation within layers VIB and V. After birth the fiber density increases rapidly. The long-range horizontal connections develop within layer II/III after the connections within layers IV–VI. But these long-range connections reach mature form long after birth: sometimes not before 15 months of age. The circuits that process local features of a visual scene therefore develop before the circuits necessary to integrate these features into a coherent neural representation of an image from the outside world.

Relevant to the issue of newborn consciousness, the connectivity of the cerebral cortex, particularly in the prefrontal area, matures later than the subcortical structures. This is corroborated by positron emission studies (PET) that have shown very low activity in the cortex except in the sensorimotor area (Chugani *et al.*, 1987). However, a recent study using fMRI has demonstrated surprisingly high resting state activity in five cortical areas (Fransson *et al.*, 2007) . The finding of high activities in the thalamus and brainstem (Chugani *et al.*, 1987) support the idea that subcortical structures are important for the control of the states of vigilance already seen during early life. The fusiform area for face recognition and the amygdala for emotions seem functional even in the newborn. As we shall see, these areas are of importance for development of social interactions (Johnson, 2005; Bassi *et al.*, 2008). In any case, concerning consciousness, these subcortical structures might not necessarily be regarded as subordinate to the cortex, particularly not in the infant (see Merker, 2007).

New methods of quantitative tensor imaging and tractography have provided images of early organization of white matter fiber bundles at birth (Dubois *et al.*, 2006; Bassi *et al.*, 2008). Interestingly, they reveal that, despite the low anisotropy of the white matter in the infant brain, most of the main fascicles described at later ages are already present at three months of age, such as corpus callosum, cerebellar peduncles, and corticospinal and spinothalamic tracts. As anticipated, asynchronies were found in the subsequent development of the infant white matter. Yet, at birth, long-range connections postulated to process the content of consciousness are, to some extent, present even though one does not anticipate a fully functional neuronal workspace.

Neurotransmitters of consciousness

Neurotransmitters such as norepinephrine and acetylcholine, which are involved in consciousness, develop progressively in the fetal brain and after birth. Norepinephrine turnover has been found to be relatively low in the rat fetus, but surges after birth (see Lagercrantz, 1996). If one extrapolates to the human newborn baby, this increased norepinephrine turnover might directly contribute to the arousal of the newborn baby, who is usually awake the first two hours after birth (see page 387).

Cholinergic basal forebrain neurons send their axons to a wide array of target structures, innervating the thalamus, hippocampus, amygdala, and cerebral cortex, and progressively develop both before and after birth. The activity of these neurons increases during awakening and have been suggested to play a critical role in the control of the states of consciousness

(Halasz, 1998; Terzano *et al*., 2000) and in promoting cortical processing of incoming stimuli. Furthermore, a rich dopaminergic innervation of the prefrontal cortex (Goldman-Rakic, 1988) accompanies cholinergic development, and cognitive advances in infants aged between 6 and 12 months have been related to this development. Neurohormones such as oxytocin may also have a role. Oxytocin has been found to reduce a rat pup's distress call when separated from its mother (Hofer, 2002) and thus is referred to as the "neurohormone of attachment" (see also Chapter 7).

Methods to study consciousness in the fetus and the infant

Consciousness cannot be assessed in infants or fetuses as easily as in adults since they are unable to report verbally the content of their subjective experience. Novel experimental approaches have thus to be developed to obviate this difficulty. A first strategy is to extend to the newborn the biophysical methods successfully used in adults for objective measurements of brain activity under conscious versus nonconscious conditions. A complementary strategy is to extend to the newborn, nonverbal cognitive and/or psychophysical tasks inspired from those used not only with human adults but also with lower mammals such as rats and mice.

Electroencephalography

Conventional EEG, amplitude-integrated EEG (see Chapter 14) and event-related potentials in the timescale of 200–400 ms can potentially be used to assess conscious versus nonconscious processing in the newborn (see Chapter 15). For example, event-related potentials in relation to visual stimuli (Johnson, 2005) may inform on both the timing of access to consciousness and the differential activation of conscious processing in "neuronal workspace" circuits.

The preterm EEG is characterized by discontinuous silent periods interrupted by "spindle-shaped bursts of fast activity" or "spontaneous activity transients" (SATs) (Vantahalo & Kaila, 2006). On the sensory side, SATs are generated already at about 24 weeks and concentrated over sensory areas. Around 30 weeks, a significant proportion become roughly coincident between hemispheres, and consistent synchrony arises at about 35 weeks, when the callosal connections become established (see Chapter 15). On the motor side, the "delta brushes" are predominantly recorded in central areas of the brain before 28 weeks. Joint recording of scalp EEG and motor activity reveals that the sporadic hand and foot movements of the fetus herald their appearance in the central (somatosensory) areas of the cerebral cortex. These findings indicate a direct relationship between the delta brushes' states of spontaneous activity and the motor behavior of the fetus and further illustrate that at these stages of development (from 28 weeks to term) sensorimotor feedback is already established in a somatotopic manner. The above-mentioned EEG activity can be reduced to ion channel dynamics of the thalamocortical neurons and the connectivity their axon weave (Llinas & Steriade, 2006). This is essential for the emergence of consciousness.

Magnetoencephalography

Magnetic signals associated with brain activity can be recorded from the newborn and the fetus in utero (Preissl *et al*., 2004; Lowery *et al*., 2006). Magnetoencephalography (MEG) possesses, like EEG, high temporal resolution (milliseconds). Furthermore it shows the response above the source area, while in the EEG, the strongest peak of the response is observed further away from the actual source in the brain. This makes the interpretation of the MEG signal straightforward in most cases, even without modeling, with respect to its approximate generator location. MEG has been combined with ultrasound and other techniques to study auditory and visual evoked responses in the newborn and even in the fetus (Huotilainen, 2006).

Functional magnetic resonance imaging

fMRI is considered to be the leading technique to explore the function of the brain (Gowland & Fulford, 2004; Seghier *et al*., 2006). fMRI has been used, for example, to assess perception of human speech in the infant (Dehaene-Lambertz *et al*., 2006). However, the fetus/infant must be immobilized and often asleep, which creates difficulties in the investigation of conscious behaviors with fMRI.

Recent fMRI mappings of prematurely born sedated infants have revealed resting-state activity in the brain (Fig. 23.3). The networks include a primary sensory area network, a posterior network including the precuneus area and lateral parietal cortex, and an anterior network encompassing the medial and dorsolateral prefrontal cortex (Fransson *et al*., 2007).

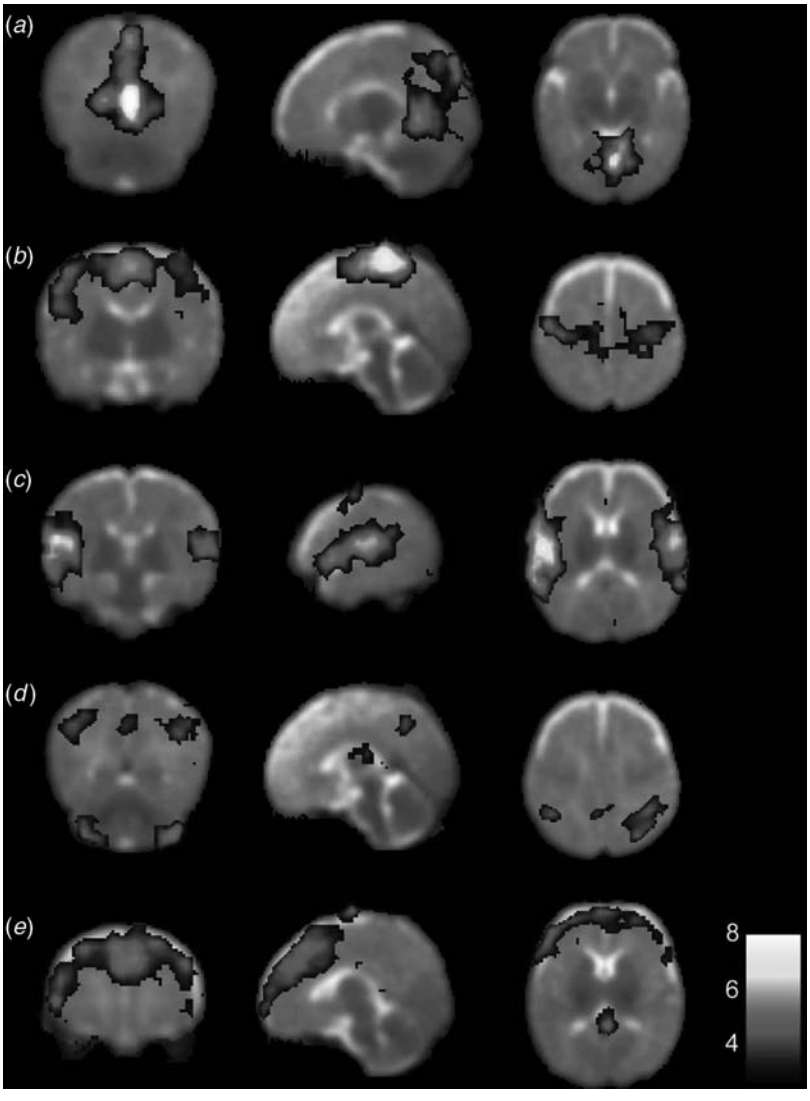

Fig. 23.3 (See color plate section.) Functional magnetic resonance imaging used to map the resting state activity of the brain in lightly sedated infants around term reveals several unique resting-state networks that encompass (*a*) the primary visual cortex, (*b*) the somatosensory and motor areas, (*c*) primary auditory cortex, (*d*) posterior lateral and midline parts of the parietal lobe and lateral aspects of cerebellum, and (*e*) the medial and dorsolateral prefrontal cortex, a network potentially of importance for access to consciousness. (From Fransson *et al.*, 2007.)

A continuing paradox is that sedated and sleeping infants respond to sensory stimuli with a decrease in the BOLD (blood oxygenation level-dependent) signal while adults respond with an increase to the same signals (Born *et al.*, 1998). However, one should be aware that the method measures changes of blood flow and that the basic mechanisms of neurovascular coupling involved may undergo qualitative changes during development.

Near-infrared spectroscopy

Near-infrared spectroscopy (NIRS) is a noninvasive, and a relatively simple and useful method that does not require immobilization. It has been used to assess how the neonatal brain processes sensory signals (Meek *et al.*, 1998). Optodes placed on the skull record near-infrared light reflected by oxyhemoglobin and deoxyhemoglobin, thus giving a measure of the hemodynamic responses over cortical areas. Algorithms have been developed to compute from the NIRS data changes in hemoglobin oxygenation, and blood volume and flow, which serve as indexes of neural activation. This method is currently used to study how sensory inputs are processed in the brain in the awake and asleep newborn. The spatial resolution is 1–2 cm and the temporal sampling resolution is 0.01 s, which is better than fMRI. One limitation is that structures located deeper than

2–3 cm under the skull cannot be studied with NIRS. With this method, positive responses to visual, auditory, and olfactory stimulation have been documented (Meek *et al.*, 1998; Bartocci *et al.*, 2000). It has also been used to study how the infant perceives human speech (Pena *et al.*, 2003).

Behavioral and cognitive tests

New behavioral paradigms might be developed based on human adult cognitive or psychophysical tasks such as tests for novelty, masking experiments, and spontaneous eye blinking (Bacher & Smotherman, 2004), and also based on attempts to assess consciousness in brain-lesioned patients (see Majerus *et al.*, 2005). Animal experiments may also offer models for simple effort-full "conscious" tasks such as delayed response tasks (trace versus delayed conditioning), flexible goal-directed behaviors and storage and recall of recent versus remote memories which have systematically identified the prefrontal cortex as playing a crucial role in these behaviors (see Changeux, 2006).

Sleep and wakefulness

Brain imaging criteria

To have access to consciousness is to be awake (Hobson, 1999). Wakefulness is often reduced to a state of brainstem and thalamic activity: a state of nonsleep-arousal. On the other hand, higher integrative functions including intentionality and self-awareness require, as discussed, higher cortical processing (Changeux & Dehaene, 2008). Also it is possible to be awake and not conscious (e.g., vegetative state) but not to be asleep and conscious. Consistent with these views, several brain imaging studies reveal that entry in the vigilant "conscious" state correlates with a progressive increase in regional cerebral blood flow, first in reticular formation followed by the thalamocortical and prefrontal-cingulate structures belonging to the "neuronal workspace" (Laureys *et al.*, 2004). This spread of activation (and deactivation) in the cerebral cortex may constitute an objective criterion for access to consciousness in the newborn infant (see Dehaene *et al.*, 2006).

Newborn full-term infants

Wakefulness of the newborn can be defined as "gross generalized body movement characterized by limb movements, prolonged startles, gross stretching, writhing and facial activity" (Stefanski *et al.*, 1984), including opening and moving of the eyes and purposeful movements of the head. This is accompanied by increased and irregular heart rate and breathing patterns. Cortical arousal is scored in parallel as an abrupt change of EEG background frequency (of at least 1 Hz) for a minimum of three seconds with a decrease of EEG amplitude and low voltage, irregular mixed pattern of EEG with frequent movement artifacts (see Chapter 14).

Fetuses and preterm infants

The exact timing for the emergence of well-defined sleep states in utero has not been definitively established. According to behavioral studies of the human fetus they appear only after 32 weeks (Prechtl, 1985). Consistent with this view, penile tumescence which is considered as an index of active sleep, together with eye movements, has been recorded in male fetuses from 36 to 41 weeks of gestation. Four-dimensional ultrasonography has further revealed at early as 22–28 weeks of development details of facial expression such as yawning, smiling, tongue expulsion, mouth and eye squeezing and blinking (Kurjak *et al.*, 2005). First of all, most of these movements are considered as from subcortical origin. Moreover, careful examination of this so called "fetal wakefulness," in particular in the sheep, suggests that it is a transition phase between active and quiet sleep (Schwab *et al.*, 2000), also referred to as "indeterminate sleep." According to these views, the fetus in utero would never have access to a vigilant conscious state (Mellor *et al.*, 2005). (Rigatto *et al.*, 1986) recorded electrocorticogram and eye and breathing movements in parallel with motor behavior monitored by video camera in fetal sheep. No wakefulness period was ever noticed in this species when analyzing video tape recordings of more than 5000 hours during eight years. In agreement with this observation, extremely preterm infants (<25 weeks) have closed eyes ex utero, consistent with the view that they are asleep (McMillen *et al.*, 1991).

This early spontaneous activity is interpreted by developmental scientists in terms rather different from the actual sleep–waking state of the adult. They basically view it as having a maturational effect. It would bring an early "inner stimulation" which would anticipate the sensorimotor experience of the newborn with the outside world (Roffwarg *et al.*, 1966; Changeux *et al.*, 1973; Meister *et al.*,

1991). This interpretation emphasizes a possible role of "sleep states" on brain synaptic plasticity since the formation of the thalamocortical relationship becomes increasingly important in the intrinsic cycles of spontaneous activity. Their role in the maturation, maintenance, and activity of the circuits concerned by the neuronal workspace processing in the newborn brain might be of critical importance (Dehaene & Changeux, 2005). Indeed, the long-range callosal connections that develop from 34 weeks onward enable a high synchrony of brain activity (Vanhatalo & Kaila, 2006) and thus the access to an emerging functional neuronal workspace close to term age.

Some criteria of consciousness

A primary requisite to be conscious is to be aware of sensory impressions, that is, the neuronal pathways mediating this information must exist and function. A distinction must be made between *sensing*, e.g., olfactory, visual, or auditory signals, and *perceiving* these sensory signals (Hepper, 1996). The latter relies on the top-down interpretation of the sensory stimuli based on previous experiences, i.e., integration of the sensory input together with stored memories in a multimodal global workspace. There are several indications that various sensory modalities are processed in the developing cortex. Spontaneous resting activity has been identified by fMRI in the primary visual areas, and the somatosensory and auditory cortices in the newborn brain (Fransson *et al.*, 2007).

Somatosensory stimuli and pain

Nociceptive reactions such as withdrawal reflexes can be recorded from the nineteenth week (Lowery *et al.*, 2007). At 20 weeks of gestation, fetuses exposed to pain have been found to have increased levels of cortisol, β-endorphin, and norepinephrine in the umbilical blood (Glover, 1999). Facial expressions similar to adults experiencing pain can be seen in preterm infants after 28 weeks (see Lee *et al.*, 2005)

The issue of the ability of the newborn and of the fetus to consciously experience pain has major consequences on the modalities of handling of newborn or premature babies in intensive care units. Two studies have been carried out recently on the assessment of somatosensory cortical activation in response to peripheral noxious stimulation using real-time NIRS (Bartocci *et al.*, 2006; Slater *et al.*, 2006). The infants in the two studies were aged between 28 and 36 weeks (40 subjects) and 25 and 45 weeks (18 subjects) of gestation and the painful stimuli were presented as either venipuncture or a heel lance. In both studies the painful stimuli produced a clear increase in hemodynamic response in the somatosensory cortex. However, in one study the response was either bilateral (in 29 newborns) or over the contralateral area (in 11 newborns) (Bartocci *et al.*, 2006) and in the second study the response was noted only in the contralateral somatosensory cortex (Slater *et al.*, 2006). The difference between the two studies was possibly due to a difference in the type of noxious stimulus (Slater *et al.*, 2006) or the variability of the neonate condition (Lowery *et al.*, 2007). A more pronounced response was seen in the youngest infants, consistent with the finding that the pain threshold is lower in preterm infants (Fitzgerald, 2005). On the other hand, there was a positive correlation between pain response and postnatal age. Interestingly, the cortical responses to noxious stimulation were found to be greater in awake than in sleeping infants (Slater *et al.*, 2006). In addition, the bilateral activation noticed in one study seemed to include regions such as S_2 cortex, anterior insula, the ventral premotor area, and the anterior cingulate cortex (Bartocci *et al.*, 2006), which have been postulated to be part of the neuronal workspace.

The available data, to date, are consistent with the notion of a minimally conscious experience of pain in the awake neonate including preterm neonates. By contrast, as discussed above, this conclusion does not seem to extend to the fetus in utero of the same age and *a fortiori* of earlier age, which are viewed as being under strong "suppressor" actions, which make them asleep or "endogenously anesthetized" (Mellor *et al.*, 2005). A likely conclusion is that pain perception strikingly differs in the awake newborn and in the fetus in utero.

Smell and taste

The behavior of alert early infants appears to be influenced by olfactory cues mainly originating from the intrauterine environment (Varendi *et al.*, 2002; Schaal *et al.*, 2004). For instance, they seem to be more attracted by the smell of amniotic fluid than by other odors. Exposure to amniotic fluid and other maternal odors have been found to have a soothing effect on newborns. Also babies exposed to

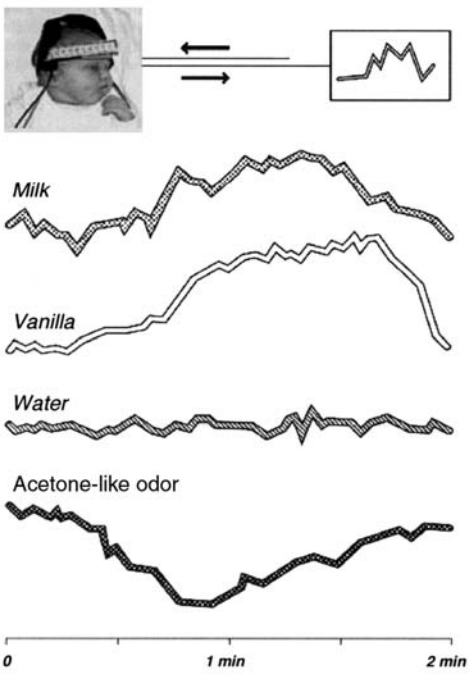

Fig. 23.4 Recording of the responses to smell by near-infrared spectroscopy (NIRS) from the olfactory cortex. This shows that various smells are processed at a cortical level. (Modified after Bartocci *et al.*, 2000.)

clothes with their own mother's odors stopped crying. Infants have also been reported to prefer tastes that they were exposed to during fetal life through their mother's diet (Schaal *et al.*, 2004). Clear behavioral responses to smell can be recorded in preterm infants from about the twenty-ninth week and the fetus can probably smell from about the twentieth gestational week, at the time the epithelial plugs blocking the nostrils disappear.

NIRS recordings (Fig. 23.4) in the left anterior orbitofrontal gyri of newborn babies in a quiet awake state show increased hemodynamic response during exposure to "pleasant" smells such as that of colostrum or of vanilla compared with water (Bartocci *et al.*, 2000). On the other hand, a decreased response, which was significantly greater on the right than on the left side, was noticed when the babies were exposed to "unpleasant" smell of a disinfectant or of a detergent (Bartocci *et al.*, 2001). However, in this study, responses were not compared between awake and sleeping babies.

In the case of the human fetus, in utero conditioning events may plausibly take place as well (Smotherman, 2002) and account for the newborn suckling behavior and possibly for the mentioned soothing effect of amniotic fluid and other maternal odors. Yet, it appears difficult at this stage to assess whether or not these smell and taste responses of the newborn are consciously experienced.

Vision, imitation, and face processing

Noninvasive visual stimulation in pregnant mothers reveals MEG fetal brain activity. Bright light was shone at the maternal abdomen for short periods (8 s) repetitively and visual-evoked responses were monitored by MEG. Activity could be recorded in the frontal eye fields but not in the primary visual cortex in the occipital region (Lowery *et al.*, 2006).

Full-term newborn infants can process complex visual stimuli, recognize faces, and imitate in spite of their low visual acuity (Johnson, 2005). They have developed preferential looking, i.e., they look longer at patterned field stimulus than at gray fields (see Chapter 12). Specialized areas of the cerebral cortex such as the fusiform face area are selectively activated by faces in the adult brain and their lesions result in prosopagnosia. A rich literature shows that infants process information about the characteristics of faces from birth (see Chapter 12). In any case, many of these face recognition experiments require the child not only to be awake and attentive but also to be sensitive to the feedback from a social factor in the *conscious* eye–contact relationship. It is not known whether face processing is also functioning in extremely preterm infants. Yet, after 26 weeks of gestation, during the short periods of wakefulness, the preterm infant is already able to briefly fix the sight of its mother (Fig. 23.5). It appears plausible that human newborns possess an inborn capacity for conscious face recognition, a characteristic element of a developing social brain network (Adolphs, 2003).

Hearing

Low-frequency sounds can be recorded from about the sixteenth week in the fetus brain (Lecanuet, 1995). However, the fetus does not react to sounds in general until the twentieth week when tachycardia can be elicited by noise (see Chapter 11). Fetal MEG responses to sounds have been recorded from fetuses between 33 and 36 weeks (Lowery *et al.*, 2006).

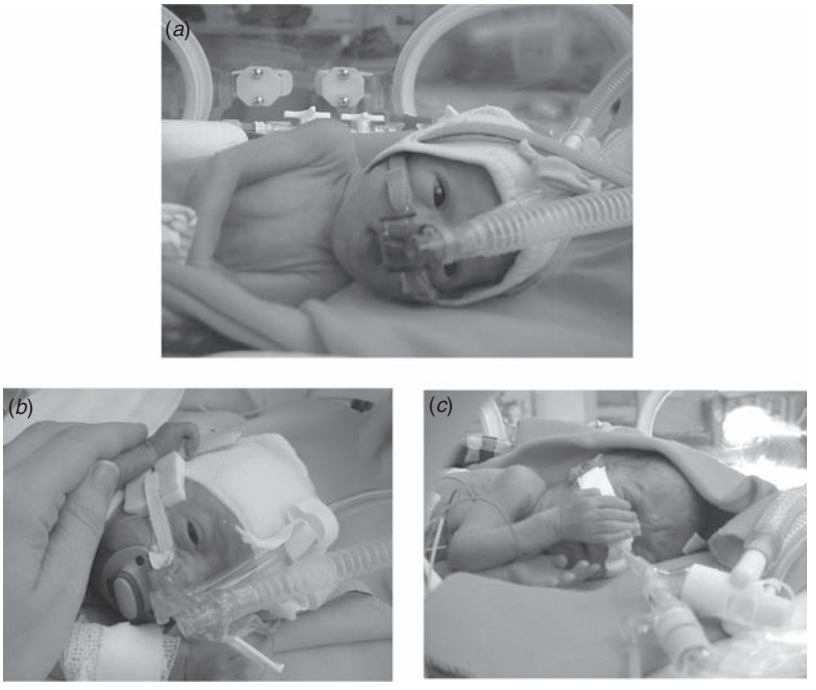

Fig. 23.5 (See color plate section.) Preterm infants of about 26 weeks are awake for short periods and seem to fix the gaze for short moments toward the mother. They also seem to be able to move their arms for protection (self-awareness). (Photos courtesy of Ann-Sofi Gustafsson.)

External sound is reduced to about half of its strength when it reaches the fetal cochlea. However, it is plausible that the maternal voice is transmitted also by direct conduction through her body. This may explain why the newborn infant seems to be able to discriminate between his or her mother and an unfamiliar woman's voice (Gray & Philbin, 2004). The full-term infant can orient visually to auditory signals by turning the head and the eyes toward the sound source. If an infant is shown, for example, an object at the same time as being presented with a sound, they will move their eyes toward the sound, suggesting that hearing is more mature at birth. On the other hand, the preterm infant seems to have difficulties in localizing an auditory stimulus.

Memory

Does the newborn remember early sensory experiences? The most primitive form of memory, i.e., habituation appears around 22–23 weeks of gestation in the human fetus. If the fetus is exposed to a repetitive stimulus such as the vibration of an electric toothbrush, it reacts with movement (Leader *et al.*, 1988). However, after multiple stimuli, it habituates to the stimulus and does not react any longer. Newborn infants remember sounds, melodies and rhythmic poems that they have been exposed to during fetal life (Hepper, 1996). However, short-term memory is rather limited in newborn infants; retention of visual objects lasts only for a few seconds. A 2-month-old baby remembers a soother or a face which suddenly disappears (Johnson, 2001).

Working memory appears around 7 months of age, according to most reviews in developmental psychology (Bauer, 2006). However, even younger babies seem to remember, for example, a soother which suddenly disappears. There seems to be some kind of mental representation of faces and things at about 2 months of age (Johnson, 2001). Thus the previously held view that out of sight is out of mind may not be true any longer for the newborn (see Chapter 12). Long-term memories disappear during early childhood (infant amnesia) and full declarative memory develops only after 3 years of age (Johnson, 2001).

Language

Human language is primarily processed in the left perisylvian regions of the human brain (Dehaene-Lambertz *et al.*, 2006). Even though language production does not become significant before the end of

the first year of life, infants display elaborate capacities for oral language perception that are rapidly modified by their linguistic environment (Kuhl, 2004). Even newborn infants can discriminate between language belonging to different rhythmic families (Nazzi *et al.*, 1998). Neonates seem to prefer rhymes that the mother has read during the last week of gestation (DeCasper & Fifer, 1980). Yet, their discrimination capacities at birth (even to sound contrasts that are not present in their environment) and which are also shared for some of them with nonhuman species, far exceed what could have been learned in utero (see Chapter 12).

Integration of multiple sensory modalities and memory

Reptiles are not able to integrate the sight and the smell of prey (Sjölander, 1999). The snake is governed by sight to strike prey, but to start to swallow the head of the prey, it must use its smell. The snake has no concept of a particular prey, e.g., mouse, and no perception of object constancy according to Piaget. Such integrative abilities may be viewed as a characteristic feature of a "global conscious workspace" that develops later in the course of evolution. Indeed, full-term human infants seem to be able to connect what they see with what they hear. One well-known example of the ability to combine sensory sensations is that newborn infants feeling pain can be calmed by sucking sucrose (Zeifman *et al.*, 1996). To achieve an optimal effect, a 4-week-old baby must also see his caregiver. This is not necessary in the 2-week-old babies, indicating that the integration of sensory inputs from different modalities have to mature. The newborn brain is able to integrate sensory signals of different modalities but not to the same extent as later in life (Morrongillo *et al.*, 1998).

Awakening at birth and the "first access to consciousness" of the newborn

The first awakening

Upon delivery, the newborn baby, who has taken an active part in its process, wakes up for the next few hours (Desmond *et al.*, 1963). The eyes are wide open with usually large pupils and it may cry. After a couple of hours it usually falls asleep again being awake the following days for only short periods of time, i.e., from 7% to 10% of the circadian cycle (Stefanski *et al.*, 1984). The delivery from the mother's womb thus would cause a *first awakening* of the infant from a "resting" or sleeping state in utero, even if the EEG signs of the cycle of sleep do not seem significantly modified by the event of birth (Vanhatalo & Kaila, 2006). The delivery stress (Lagercrantz & Slotkin, 1986) together with the transition from the aqueous and warm environment of the mother's womb into an hostile, aerial, and cold outside world is, in reality, expected to have dramatic consequences on the actual state of consciousness of the newborn and its electrophysiological signs in particular due to an intense flow of novel sensory stimuli. The internal state of the "neuronal workspace" mostly driven by the above mentioned SATs in utero may suddenly switch to a sensory controlled regimen, which would correlate with the observed marked decrease of the amplitude of SATs in scalp EEG recordings (Vanhatalo & Kaila, 2006). Moreover, the circadian rhythms of quiet and active sleep which are maternally influenced in utero, together with the incorporation of periods of wakefulness, become progressively circadian again after birth. In the preterm infant, it is expected that the actual access and consequences to the "first awakening" may significantly vary with the state of maturation of the brain. Moreover, it is not anticipated that the <25-week preterm infant, in whom the thalamic connections have not yet penetrated the cortical plate and where callosal connections have not been established, have access to a first awaking state comparable with that of the full-term newborn.

The stress of being born

The stress from vaginal delivery together with the multiple and strong sensory stimulations caused by the physical contact with an oxygenated and cold extrauterine world, might be critical in the awakening of the newborn and the accompanying first cry. There is probably a parallel activation of the cerebral norepinephrinergic system from the locus ceruleus and increased norepinephrine turnover leading to the first authentic awakening of the newborn and to its first breaths (see Chapter 7). During the stress

of being born, the cholinergic system may be activated as well. Neurons of the rostral ventrolateral oblongata (RVLM) provide one of two major sources of afferent inputs to the locus ceruleus. Acetylcholine would thus be an important neurotransmitter in the control of the states of consciousness (see Koch, 2004; Changeux, 2006), a possibility corroborated by the finding of increased acetylcholine release during awakeness.

Blocking the activation of the monoaminergic and cholinergic systems may, in rodent pups, blunt the arousal response to hypoxia and increase the mortality. In a similar way, mice that lack β_2-containing nicotinic acetylcholine receptors lack the ability to arouse to the same extent as wild-type mice and a similar phenotype is observed in newborn pups after chronic exposure of the pregnant mother to nicotine (Cohen *et al.*, 2002, 2005). These animal models have been used in the study of the mechanisms of sudden infant death syndrome, which may be regarded as a disease with sudden loss of awakening and breathing. The available data are consistent with the notion that nicotinic acetylcholine receptors are involved in the phasic expression of arousal promoting mechanisms (Lena *et al.*, 2004).

The first breaths

The first breaths and the subsequent increase of oxygen in the blood may also contribute to the awakening of the newborn infant. The spirit or the pneuma inhaled by the newborn refers to the commencement of human life, i.e., the access to consciousness. "Words are breathing" screams Hamlet's mother Gertrud. For (Denton, 2005), the hunger for air is a primordial emotion. Before birth, pO_2 is low and may reduce the activity of the brain possibly via neuromodulators such as adenosine (Irestedt *et al.*, 1989). Also the expiration of carbon dioxide, which is slightly elevated in the fetus, may stimulate the activation of the brain. Thus, the first breaths of air may contribute to the arousal of the newborn baby.

The first awakening also releases an inborn "positive emotion," a "motivation" oriented toward the outside world and in particular the feeding mother. It is interesting to note that in many species, this first awakening drives the newborn to spontaneously explore the world, looking in particular for food, to the extent that Denton (2005) views the occurrence of such primordial emotion as marking the dawning of consciousness in the evolution of animal species. As pointed out by Trevarthen and Aitken (2001) human infants have a multiplicity of goals engaging both the social and inanimate environment such as interacting with others and acting on objects together with internal goals such as maintaining homeostasis. The emotions of the newborn infant are assumed to play a critical role in the evaluation of the fulfillment of their goals. The infant affective display then becomes part of a conscious inter-communication system with the caretaker (Tronick, 1989) where, as we shall see, crying plays a critical role.

Crying, self-awareness, and social interactions in the newborn

Crying

An almost unique feature of the human newborn is that following the first awaking, it cries. Crying can be regarded as a distinct state of consciousness (Giganti *et al.*, 2006). Crying has recently been reviewed systematically (Soltis, 2004). Sucrose is well known to have a quietening effect on crying infants and is commonly used to calm infants during painful procedures (Zeifman *et al.*, 1996). This effect can be blocked by naloxone, indicating that endorphins might as well be involved. This also suggests that the undifferentiated crying may express a sign of discomfort of the newborn resulting from the fast change of environment consecutive to delivery and the necessary adaptation of the vital parameters to aerial life. Crying thus appears as a transient state of consciousness essential for the survival of the particularly immature newborn infant through the establishment of efficient social interaction with the mother.

Self-awareness

The newborn infant at birth already reacts differently to tactile stimulation by the mother as compared with self-stimulation that he or she does not respond to (Rochat, 2003). The newborn infant is known to imitate certain body movements. Tongue protrusion by an adult will stimulate tongue protrusion in an awake infant (Meltzoff & Moore, 1977). Other forms of imitation of facial expressions have been reported. Yet, their interpretation as imitations has been challenged (Anisfield *et al.*, 2001) and they are often preferably referred to as "matching behavior" (Jacobson, 1979).

Empathy of infants with their caring parents through crying

Empathy develops much earlier than mentalizing abilities or theory of mind in the newborn. Indeed, as a consequence of affect sharing, emotional contagion may develop, and examples for such contagion can be observed even in the newborn. First of all they distinguish their own cry from the cry of another newborn, and second, they respond significantly more with crying when hearing another newborn crying than when hearing their own cry, the cry of an older baby, or a synthetic cry (Decety & Jackson, 2004). No brain imaging studies have yet been carried out under such conditions of early empathetic social exchange.

Recently, preliminary attempts have been made to image brain activity in crying infants and in their caring parents (Laureys, 2004). Using EEG, right frontal activation asymmetry is already noticeable in 1-month-old infants, related to more frequent sad and pre-cry faces (Jones *et al.*, 1997). Infants who cried in response to maternal separation had greater right frontal asymmetry compared with infants who did not cry during the preceding baseline period (Davidson & Fox, 1989). Neural signs of negative emotion accompanying crying may thus be recorded in neonates. Similarly, disagreeable stimuli, e.g., noxious heel stroke (Fernandez *et al.*, 2003), causes right or left frontal activation.

Soltis (2004) has suggested that the newborn's cries mobilize neuronal networks in the parent brain that may elicit positive caregiving, for instance to satisfy hunger. The neural correlates for a conscious social communication of the newborn with their caregiver through crying should now be accessible to scientific investigation. We can hypothesize that the neuronal workspace is involved in this intersubjective communication.

Conclusions

The fetus in utero is almost continuously asleep and unconscious partially due to endogenous sedation, in particular it would not consciously experience nociceptive inputs as pain. On the other hand, the *newborn infant* exhibits in addition to sensory awareness – especially to painful stimuli – the ability to process memorized mental representations, to differentiate between self and nonself touch, sense that their bodies are separate from the world, to express emotions, and to show signs of shared feelings. Moreover "objective signs" for the mobilization of the global neuronal workspace circuits are being detected – still in a rather limited manner – in awake infants at the level of the prefrontal cortex in sensory processing, in responses to novelty and to speech and in social interaction. Yet, the newborn's capacities for internal manipulations in working memory are reduced, it is unreflective, present oriented, and makes little reference to concept of him or herself. Newborn infants display features characteristic of what may be referred to as *basic consciousness* (Changeux, 2006). They still have to undergo considerable maturation to reach the level of adult consciousness (Zelazo, 2004).

The *preterm infant* ex utero, may open its eyes and establish minimal eye contact with its mother. It also shows avoidance reactions to harmful stimuli. The connections with the workspace circuits are not yet fully established. Our view is that it has reached only a lower level of *minimal consciousness* analogous (though of course not identical) to that of a rat or mouse. A pending question is the status of the preterm fetus *born before 26 weeks* (<700 g): these neonates have closed eyes and seem constantly asleep. The immaturity of their brain networks is such that it may not even have reached a level of minimal consciousness. The postnatal maturation of the brain may be delayed (Dubois *et al.*, 2008) and there are indications that the connectivity with the global neuronal workspace will be suboptimal (Kapellou *et al.*, 2006) in some cases, as indicated by deficient executive functions (Marlow *et al.*, 2005; Lagercrantz, 2007). The timing of the emergence of minimal consciousness has been proposed as an ethical limit of human viability.

Studying the brain and its functions from early fetal stages in utero up to the adult state, in any case, offers fruitful and promising insights into the understanding of the neural bases of consciousness.

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