Stem Cell Biology and Regenerative Medicine

Marie-Pierre Junier
Steven G. Kernie *Editors*

Endogenous Stem Cell-Based Brain Remodeling in Mammals

Humana Press

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Series Editor
Kursad Turksen, Ph.D.
kturksen@ohri.ca

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Editors
Marie-Pierre Junier, Ph.D.
French Institute of Health
and Medical Research U1130
French National Center
for Scientific Research
Pierre-and-Marie-Curie University
Paris, France

Steven G. Kernie, M.D.
Departments of Pediatrics and Pathology
& Cell Biology
Columbia University College of Physicians
and Surgeons
New York, NY, USA

ISSN 2196-8985
ISBN 978-1-4899-7398-6
DOI 10.1007/978-1-4899-7399-3
Springer New York Heidelberg Dordrecht London

ISSN 2196-8993 (electronic)
ISBN 978-1-4899-7399-3 (eBook)

Library of Congress Control Number: 2014931105

Printed on acid-free paper

Humana Press is a brand of Springer
Springer is part of Springer Science+Business Media (www.springer.com)

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Contributors

Alison L. Althaus Department of Neurology and Neuroscience Graduate Program, University of Michigan, Ann Arbor, MI, USA

Stéphane Auvin French Institute of Health and Medical Research U676, Paris Diderot University, Sorbonne Paris Cité, PremUP, Paris, France

Praveen Ballabh Department of Pediatrics, Cell Biology, and Anatomy, Regional Neonatal Center, Maria Fareri Children's Hospital, Westchester Medical Center-New York Medical College, Valhalla, NY, USA

Olivier Baud French Institute of Health and Medical Research, U676, Paris Diderot University, Sorbonne Paris Cité, PremUP, Paris, France

Vibol Chhor French Institute of Health and Medical Research, U1141, Paris Diderot University, Sorbonne Paris Cité, PremUP, Paris, France

Isabelle Dusart Pierre-and-Marie-Curie University – Paris 6, French National Center for Scientific Research, UMR 7102, Paris, France

Elias A. El-Habr French Institute of Health and Medical Research, U1130, French National Center for Scientific Research, Pierre-and-Marie-Curie University, Paris, France

Bobbi Fleiss French Institute of Health and Medical Research, U1141, Paris Diderot University, Sorbonne Paris Cité, PremUP, Paris, France

Pierre Gressens French Institute of Health and Medical Research, U1141, Paris Diderot University, Sorbonne Paris Cité, PremUP, Paris, France

Sue Hong Departments of Pediatrics and Pathology & Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY, USA

Marie-Pierre Junier French Institute of Health and Medical Research, U1130, French National Center for Scientific Research, Pierre-and-Marie-Curie University, Paris, France

Steven G. Kernie Departments of Pediatrics and Pathology & Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY, USA

Nathalie Lefort French Institute of Health and Medical Research, UMR 861, I-Stem, French Muscular Dystrophy Association, Institute for Stem Cell Therapy and Exploration of Monogenic Diseases, University of Évry Val d'Essonne, Evry Cedex, France

Jean-Marie Mangin French Institute of Health and Medical Research, U952, Pierre-and-Marie-Curie University, Paris, France

Sergio R. Ojeda Division of Neuroscience, Oregon National Primate Research Center-Oregon Health and Science University, Beaverton, OR, USA

Jack M. Parent Department of Neurology and Neuroscience Graduate Program, University of Michigan, Ann Arbor, MI, USA

Marc Peschanski French Institute of Health and Medical Research, UMR 861, I-Stem, French Muscular Dystrophy Association, Institute for Stem Cell Therapy and Exploration of Monogenic Diseases, University of Évry Val d'Essonne, Evry Cedex, France

Vincent Prevot French Institute of Health and Medical Research, Jean-Pierre Aubert Research Center, Development and Plasticity of the Postnatal Brain, Unit 837, University of Lille 2, School of Medicine, Lille, France

Ariane Sharif French Institute of Health and Medical Research, Jean-Pierre Aubert Research Center, Development and Plasticity of the Postnatal Brain, Unit 837, University of Lille 2, School of Medicine, Lille, France

Constantino Sotelo Sorbonne University, Pierre-and-Marie-Curie University – Paris 6, French Institute of Health and Medical Research, UMR S 968, French National Center for Scientific Research, UMR 7210, The Vision Institute, Paris, France

Luigi Titomanlio French Institute of Health and Medical Research, U676, Paris Diderot University, Sorbonne Paris Cité, PremUP, Paris, France

Tzong-Shiue Yu Departments of Pediatrics and Pathology & Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY, USA

Chapter 1
Introduction

Marie-Pierre Junier and Steven G. Kernie

The remarkable progress made over the last decade in our understanding of and ability to manipulate embryonic and somatic stem cells has led to the very real potential of cell-based therapies as a compelling choice for regenerative medicine. In the central nervous system, where cellular specialization is so diverse, this mode of therapy is particularly attractive. However, as more is becoming known about the intrinsic repair capabilities of the brain, strategies for harnessing the innate repair potential from endogenous progenitors are beginning to emerge. Studies to date indicate that the mobilization of endogenous neural stem cells or progenitors could also be of benefit in the development of regenerative strategies. This is especially true in children who have long been known to exhibit a remarkable ability to partially compensate from brain damage of various origins. In this book, we highlight the endogenous regenerative potential of the central nervous system in neonates and juveniles and discuss possible ways it might be manipulated for medical purposes.

The notion of brain plasticity has been around for many decades. In the 1960s, Altman provided compelling evidence that would ultimately overturn the long-held dogma that neurogenesis occurred exclusively during prenatal periods, and that the ability to generate new neurons simply did not exist in mammals (Altman 1962, 1963). Unfortunately, these observations that occurred 50 years ago were mainly ignored or lost until the 1980s when there was a resurgent interest in neurogenesis that started in the context of adult songbirds (Goldman and Nottebohm 1983). Since then, there have been countless studies into what is now moving beyond interesting

M.-P. Junier, Ph.D. (✉)
French Institute of Health and Medical Research U1130, French National Center for Scientific Research, Pierre-and-Marie-Curie University, Paris, France
e-mail: marie-pierre.junier@inserm.fr

S.G. Kernie, M.D. (✉)
Departments of Pediatrics and Pathology & Cell Biology,
Columbia University College of Physicians and Surgeons, New York, NY 10032, USA
e-mail: sk3516@columbia.edu

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3_1,

phenomenology—the recognition that neurogenesis is ongoing throughout life in discrete areas of the mammalian brain, and that this ongoing neurogenesis may functionally contribute to learning and memory.

Here, we address not simply neurogenesis in the context of normal development but instead focus on both the neurogenesis and gliogenesis that occur in response to injury. In particular, we review the studies that have occurred in younger animals where there are a variety of plasticity windows and unique circumstances that make the developing brain both more amenable to some aspects of plasticity and at the same time more vulnerable to others.

The first section (Chaps. 2 and 3) starts with Sotelo and Dusart who take a wide-ranging historical overview on what has been learned over the last centuries regarding structural plasticity within the mammalian brain. This includes both developmental plasticity and an introduction to transplant biology within the central nervous system, and how what we have learned from these experiments directs our understanding of injury-induced plasticity. This is followed by a chapter by Fleiss et al., who look at how injured tissue influences brain plasticity, particularly in the context of stem cell fate.

The next section explores neurogenesis particularly in the context of injury (Chaps. 4 and 5) and also highlights the more recently characterized neurogenic zone within the hypothalamus (Chap. 6). Hong, Yu, and Kernie provide emerging evidence that suggests injury-induced neurogenesis following traumatic, hypoxic, and stroke-related injuries appears to direct functional neurogenesis that may play a role in the spontaneous recovery seen following these oftentimes devastating injuries. This is followed by an intriguing overview from Althaus and Parent who explore how seizures are related to neurogenesis and may play a role in the development of certain kinds of epilepsy, highlighting how injury-induced neurogenesis may be harmful. Next, Sharif, Ojeda, and Prevot look at hypothalamic neurogenesis, a relatively new player in postnatal neurogenesis where they provide further evidence about plasticity that is just beginning to be explored in depth.

The next two chapters (Chaps. 7 and 8) examine gliogenesis and gliogenic progenitors both during development and in response to injury. Mangin provides a compelling overview about the regenerative potential of NG2 cells and explores how these cells represent a subtype of progenitors that possess a vast array of potential fates following various kinds of injury. Next, Ballabh looks particularly at oligodendrocyte progenitors and how these cells are highly state-dependent. The neurological sequelae seen following blood-brain barrier disruption in general and prematurity-associated intraventricular hemorrhage in particular lead to irreversible brain damage in premature infants that is difficult to mitigate even in the presence of vast numbers of progenitors.

Finally, the last two chapters (Chaps. 9 and 10) explore in depth some of the emerging concepts that temper some of the enthusiasm associated with potential therapies using endogenous stem cells. Lefort and Peschanski provide a timely and thorough look at the challenges that accompany the promise and speculation surrounding induced pluripotent stem cells (iPSCs) and their potential to provide CNS repair following injury. Many of these challenges are seen in the genomic instability

that is inherent in these cells. These challenges are then highlighted in the final chapter from El-Habr and Junier who draw parallels from brain tumor initiation and how stem and progenitor populations are more likely to undergo malignant transformation due to their underlying similarity with many aspects of cancer formation.

While this compilation of works addressing endogenous reparative mechanisms seen after brain injury is in no way complete, it will hopefully provide the reader with a compelling coherent overview of how our understanding of brain plasticity has advanced over the past decades. In addition, it should also highlight the challenges that lay ahead in our ability to therapeutically harness this potential without causing additional harm. It is only when these are balanced can we meaningfully approach stem cell-based therapy following severe brain injuries.

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Chapter 2
Structural Plasticity in Adult Nervous System: An Historic Perspective

Constantino Sotelo and Isabelle Dusart

Abbreviations

6OHDA	6-Hydroxydopamine
AChE	Acetylcholinesterase
AIS	Axonal initial segments
ChABC	Chondroitinase ABC
CLSM	Confocal laser scanning microscopy
CNS	Central nervous system
CST	Corticospinal tract
DA	Dopamine
E	Embryonic day
LTP	Long-term potentiation
MFB	Medial forebrain bundle
P	Postnatal day
PC	Purkinje cell
Pcd	Purkinje cell degeneration mutant strain of mice
PSDs	Postsynaptic differentiations
TEA	Tetraethylammonium
TPLSM	Two-photon laser scanning microscopy

C. Sotelo (✉)
Sorbonne University, Pierre-and-Marie-Curie University – Paris 6, French Institute of Health and Medical Research, UMR S 968, French National Center for Scientific Research, UMR 7210, The Vision Institute Paris, Spain
e-mail: constantino.sotelo@inserm.fr

I. Dusart
Pierre-and-Marie-Curie University – Paris 6, French National Center for Scientific Research, UMR 7102, Paris, France

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3_2, © Springer Science+Business Media New York 2014

2.1 Introduction

The term plasticity was introduced in biology in the middle of the nineteenth century, referring to the adaptability of an organism to changes in its environment (Jones 2000). In the field of neuroscience, even though neuroscientists are very much familiar and commonly used the noun "plasticity," they do not share a common definition. The term plasticity is a source of misunderstanding because of its unreliability. Indeed, plasticity does not have the same meaning depending on the context it is used, for instance, plasticity of phenotypic expressions, synaptic plasticity, morphological or functional plasticity, plasticity of sensorimotor coordination, behavioral plasticity, and postlesion plasticity. As questioned by Jacques Paillard (1976), page 33: "**Are the realities covered by this term sufficiently precise so that we can hope to see it to become a heuristic concept generator of new hypotheses and new experiences, and thus a useful concept in neurobiology?**"

It is not our intention to provide a long summary of the history of all concepts covered by the generic name neuroplasticity. On the contrary, as neuromorphologists, the focus of the present chapter will be very much reduced to morphological aspects of the plasticity, and many of our examples are taken from the cerebellum. We know that the review is far from being complete. There are whole sections that are not even mentioned such as developmental plasticity, critical periods, and phylogenetic aspects of the neural plasticity. Finally, the specific case of neural stem cell and its potential interest in nervous system repair have been treated in other chapters of this book or in numerous recent reviews (see, for instance, in Martino et al. 2011; Saha et al. 2012).

2.2 Origin of the Term "Plasticity"

2.2.1 The Importance of Psychologists in the Development of the Plasticity Concept

From his earliest origins, man has been interested in understanding the world around him and the nature of his constant interactions with that world. Because man has rapidly intuited that such interactions were possible, thanks to the interface that represents the brain, the mystery of how the nervous system was built and how it functions has passionately and simultaneously disturbed the human being. The aphorism "know thyself" has been the incentive that has driven scientists of all ages to try to unravel the mysteries of brain function.

One of the most amazing abilities of the brain is the mental process that allows us to learn and to keep in memory the learned tasks. During the second half of the nineteenth century, with the earliest scientific studies on learning and memory, it became obvious that during all his life the human being was able to learn and to

remember and that the brain was the receptacle to accumulate new knowledge. The brain should be therefore provided with some kind of mechanism capable of fixing and preserving constant acquisitions. Due to the very poor knowledge of the brain constitution, organization, and function, a number of speculations began to appear trying to imagine how the brain was able to work. It is of interest to recall that the more accurate and fertile imagination appeared among psychologists. At the middle of the nineteenth century, Alexander Bain (1818–1903), an "associationist psychologist," who tried to endorse psychology with experimental sensorimotor physiology, published his textbook, entitled "The Senses and the Intellect" (1855) where he started to expose his ideas on the possible mechanisms of memory. His devise was "**There is nothing I wish more than so to unite psychology and physiology**." These ideas were completed in another book, "Mind and Body: The Theories of Their Relation," published 18 years later, where he explained in Chap. 5, devoted to the intellect, that the mechanism of retention would pass through the growth of the number of cell junctions between the nerve cells, establishing precise new neural groupings. Using his own words, Bain wrote on page 91: "**For every act of memory, every exercise of bodily aptitude, every habit, recollection, train of ideas, there is a specific grouping, or co-ordination, of sensations and movements, by virtue of specific growths in the cell junctions. For example, when I see a written word and, as a result of my education, pronounce it orally, the power lies in a series of definite groupings or connexions of nerve currents in the nerve and centres of the eye, with currents in motor nerves proceeding to the chest, larynx and mouth; and these groupings or connexions are effected by definite growths at certain proper or convenient cell crossings**".

What was the meaning of "cell junctions" or "cell crossing" in the central nervous system when Bain wrote the abovementioned sentences? In his publications, Bain provided descriptions of the organization of the nervous tissue based primarily upon the histological studies of Lionel S. Beale (1828–1906) who considered, like many others of his contemporaries, that the nervous system was structurally continuous.

Alexander Bain (1873, pp. 118–119) summarized Beale's ideas published in 1862 and 1863, as following: "**The manner of connexion of the nerve-fibres […] is conjectured and figured by Dr. Beale in a plan that facilitates our conception of the physical growths underlying memory and acquisition […] He observed, […] a series of hues passing across the body of the cell, and continuing into its branches, or communicating with the nerves. He considers these lines as the tracks of nervous action through the cell […] He couples with, this appearance the doctrine (maintained by him, although disputed by others) that the nerves terminate in loops, and consequently form an unbroken nervous circuit. He then suggests that the cell-crossing is the place where the inner bendings of a great many independent circuits come into close neighbourhood, and affect one another by a process of the nature of electrical induction. Any one of the circuits being active, or excited, would impart excitement to all that came near it in the same cell**".

It is, therefore, evident that the knowledge on the organization of the central nervous system gathered by Bain was inadequate. Although Beale published his morphological work 8 years before Gerlach (1871), their conception of the organization of the CNS (central nervous system) was similar. Therefore, the description of Beale was of the same nature than the wrong hypothesis later on enunciated by Joseph von Gerlach (1820–1896), who with a flawed gold-impregnation method decided that the fibers (axons and dendrites) emerging from different cells fused and formed a large interlocking plexus or network that occupied the whole of the gray matter ("reticular theory" or "reticularism"). Within this framework and knowing that the nerve impulses were electrical in nature, it is not difficult to imagine what the notion of Alexander Bain of a "cell junction": the points of fibers intercrossing, considered like the specific sites for cell-to-cell interactions. In any case, in agreement with Stanley Finger (1994), Bain should be considered like one of the pioneers in postulating that the malleability of the nervous system—in what is referring to memory and learning—must go through structural changes in its circuits and, more importantly, that nerve activity should be responsible for such changes. The ancient metaphor that the brain was like a wax tablet, in which you could write or delete messages, began to have its biological counterpart.

Almost simultaneously, another psychologist William B Carpenter (1813–1885) in his book "Principles on Mental Physiology, with Their Applications to the Training and Discipline of the Mind and the Study of Its Morbid Conditions" (1874) used the term "plastic" on a quite similar sense, although no attempts to explain the mechanisms were done. In the chapter related to Habits (Habit of Self-discipline), Carpenter wrote page 289: "**Whilst, then, every one admits the special strength of those *early impressions* which are received when the Mind is most 'plastic,'—most fitted to receive and retain them, and to embody them (as it were) into its own Constitution, […] *shaping* that Mechanism, whose subsequent action mainly determines our Intellectual and Moral character, and, consequently, the whole course of our conscious lives**".

Somewhat more perspicacious was William James (1842–1910), who in his book on "The Principles of Psychology" (Vol. 1, Chap. 4 devoted to Habit, 1890) provided one of the earliest descriptions of the plastic nature of the nervous system and the changes of its circuits associated with the foundation of habits. He wrote on page 105: "**The change of structure […] may be invisible and molecular, as when a bar of iron becomes magnetic or crystalline through the action of certain outward cause. […] Plasticity, then, in the wide sense of the word, means the possession of a structure weak enough to yield to an influence, but strong enough not to yield all at once. […] Organic matter, especially nervous tissue, seems endowed with a very extraordinary degree of plasticity of this sort; so that we may without hesitation lay down as our first proposition the following, that the phenomena of habit in living beings are due to the plasticity of the organic materials of which their bodies are composed**".

James, though aware of his ignorance on the real nature of the morphological substrate providing plasticity to the brain, described the nerve impulses as responsible for the induction of the plastic changes. However, the search of brain anatomy

and cellular organization does not receive the required attention. Despite the importance of his assumptions and interpretations, James succumbed to the main weakness of those days' psychology, when the CNS was simply regarded as a black box and only the quality of incoming and outgoing messages was of interest and not what might happen inside the box. Despite this weakness, he correctly understood that nervous activity was responsible for the elaboration of new paths within the CNS, as he put it on page 107: "**If habits are due to the plasticity of materials to outward agents, we can immediately see to what outward influences, if to any, the brain-matter is plastic. […] The only impressions that can be made upon them are through the blood, on the one hand, and through the sensory nerve-roots, on the other; and it is to the infinitely attenuated currents that pour in through these latter channels that the hemispherical cortex shows itself to be so peculiarly susceptible. The currents, once in, must find a way out. In getting out they leave their traces in the paths which they take. The only thing they can do, in short, is to deepen old paths or to make new ones; and the whole plasticity of the brain sums itself up in two words when we call it an organ in which currents pouring in from the sense-organs make with extreme facility paths which do not easily disappear**".

Further in the book, when James developed his ideas about the concept of plasticity (page 566), he wrote: "**Let us then assume as the basis of all our subsequent reasoning this law:** ***When two elementary brain-processes have been active together or in immediate succession, one of them, on reoccurring, tends to propagate its excitement into the other***".

Envisaging that the simultaneous activity might not only reinforce the interactions between excited neurons but also create new interactive paths. Therefore, in spite of his poor anatomical vision of the organization of the brain, James advanced in his promulgated law a concept that, if it is interpreted by our current knowledge, turns out to be prophetic since it foresees the one of the "Hebbian synapses" as defined by Donald Hebb in 1949, almost 60 years later (see below). It is, therefore, justified that in many papers dealing with the history of neuroplasticity, William James is considered to be the first researcher in using the term plasticity from a modern perspective (Berlucchi and Buchtel 2009).

2.2.2 The "Neuron Doctrine" and Its Corollary, the Synaptic Contacts, Provided a Favorable Correlate to Explain Neuroplastic Changes

The difficulty to envision a more anatomical solution to the problem raised by the understanding of the learning and memory mechanisms began to be dispelled 2 years before the publication of James' book, when Santiago Ramon y Cajal (1852–1934) started the study of the organization of the cortex of the cerebellum with the Golgi impregnation. In this study, practically completed in 3 years (Cajal 1988–1890),

Cajal not only revealed the organization of the cerebellar circuits, but the study had much more general implications. Indeed, the presence of basket cells, with their peculiar pericellular baskets and "pinceaux formations" (brushlike), together with the correct interpretation of the developmental history of the climbing fibers, and their ultimate disposition coiling up along the main branches of the Purkinje cell (PC) dendrite (see in Sotelo 2008), incited Cajal to conceive that the diffuse nerve network supposed by Gerlach (1871) and later on (1873) by Golgi (1843–1926) did not exist. On the contrary, axons—as it was the case for dendrites—ended freely. A few years later, Cajal (1892) formulated his "neuron doctrine" stipulating that between neuronal processes there was no continuity, only contiguity. Within this framework, the CNS was composed of individual cells, like all the other tissues of the body, communicating between them through specific contact zones (the nervous articulations of Cajal), later on (1897) coined synapses by Sherrington (1857–1952). From now on, a new path was opened for a better comprehension of the mechanisms involved in the malleability of the nervous tissue during the processes of learning and memory and for a better definition of neuronal plasticity.

The newly opened path was rapidly taken by Eugenio Tanzi (1856–1934), one of the earliest and enthusiastic defenders of the "neuronal doctrine" proposed by Cajal, who in 1893 had the intuition that nerve current should find some resistance at the passage from one neuron to the next, throughout their cell junctions. The stretching of the arriving axon and/or the receiving dendrite, when the cell junctions are repetitively stimulated, similarly to what happens when exercising muscle fibers (hypertrophy), might overpass this resistance. This distance reduction would be the plastic change required for the phenomenon of learning through the facilitation of the passage of nerve impulses. In Tanzi's words (1893), page 469: "**If now we think that the interposed distance between the terminal arborisation of one neuron and the body of the next neuron constitute a resistance or […] a kind of difficult passage ('mal passo') that the nervous wave must overcome not without difficulty, it is evident that the conductibility of the nervous system must stand in an inverse relation of the interval between the two neurons. To the extent that exercise has the tendency to shorten the distances, it increases the conductibility of the neurons that is their functional capacity**" (taken from the English translation published in the paper by Berlucchi and Buchtel 2009).

Another important actor in the history of what from now on we can call "synaptic plasticity" was Ernesto Lugaro (1870–1940), a clinical physician and psychiatrist. Between 1898 and 1906, when he published his treatise on psychiatry (translated to English in 1909), Lugaro expanded Tanzi's hypothesis and coined the term "plastic activity of neurons." Thus, after summarizing Cajal's anatomical ideas, he wrote pages 94–95: "**Let us now see if we can draw some deductions in psychology from these data regarding the anatomy and general physiology of the nervous system […] One of the problems which psychology has always put to anatomy is that of interpreting psychic evolution during development and adult life […] Anatomy gives us the general impression that organs are systems of unchangeable and rigid structure. How then can psychic plasticity be reconciled with this rigidity? The neurone doctrine is capable of giving us a satisfactory answer**

if we admit that between neurone and neurone there is some kind of constant or temporary discontinuity, no matter whether the relationship is established by contact or by means of plastic connections which undergo modifications most readily."

It is remarkable how Lugaro explained that the nervous system could be considered like a kind of "neoteny" (a process during which some developmental features are preserved in adulthood). For him, adult life is simply a continuation of development ("**If then we consider that the psychic maturity acquired in adult life is simply a continuation of development itself, the explanation will appear more clear and convincing to us**."), and, therefore for the brain, aging would be only a slowed down developmental processes. Finally, as clearly addressed by Berlucchi and Buchtel (2009), Lugaro succeeded in making a bridge between Tanzi's modifiability of synapses and Cajal's neurotropism, focusing again on the synapses as the main location of the neuronal plasticity, pages 96–97: "**Neurotropism […] does not exclude the possibility that in certain organisms the connections between the various neurones are immutable […] Probably this holds in the case of the invertebrates. But where the structure of the nerve centres, although already highly organised is still capable of further perfection—especially in the cerebral cortex—it is clear that chemotropic activities can still be the factor in bringing about new anatomical relationships. Tanzi formerly explained the consolidation of memories and automatic actions which become habitual, by means of an ever expanding mechanism which tends to approximate and unite in a progressively more intimate manner elements connected with each other […] This plastic activity of the neurones, which is just a continuation of what is more accentuated in the embryo though in quite a different manner, is naturally more active in infancy and youth than in adult life. And that explains the particular plasticity of the young mind which education models so easily. To the same cause can be attributed the fact that after destruction of some cortical centres functional compensation—impossible in the adult—is possible in the child […] With the increase of years the plasticity of the neurones becomes quiescent, but very slowly, and in fact we see how the aged may become incapable of modifying their own convictions and of yielding to the suggestion of new experiences**."

2.2.3 Cajal's Writings on Neuroplasticity

In a review concerning the drawings of Cajal, Sotelo wrote about the pros and cons of his inspiring role on the development of the neurosciences since 1888 (Sotelo 2003). Indeed, from his first review (Cajal 1892) about the histology of the nervous centers, he always tried to link his structural discoveries with their possible functional meaning. Although he was prophetic in many of his functional assumptions, sometimes he changed opinion during his long life of work, and other of his assumptions turned out to be wrong (Sotelo 2003). One of these undecided topics is the one

of neural plasticity. After his deep morphological analyses of the degeneration and regeneration of the nerve fibers (Cajal 1913/1914), Cajal was stressed by the fact that peripheral axons regenerate while central axons were only able of abortive regeneration. This essential disparity results from differences in the cellular and molecular environment, making the CNS a hostile milieu for axon growth. Moreover, during his early years of research as a neuroembryologist, Cajal—together with Wilhelm His (1886, 1889), Camillo Golgi (1873), and Alfred Kölliker (1896)—reached the conclusion that differentiated neurons do not divide and only neuroblasts were able of mitotic activity. Since the latter are only present in developing periods, the CNS of adult mammals had completely loss their capacity to generate new neurons. These two important observations prompted Cajal (1928) to deduce that: "**In the adult centers the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated**." This sentence has been taken by many authors as the irrefutable proof that Cajal was not a supporter of neuroplasticity.

However, after the publications of Jones (1994), DeFelipe (2006), and many others, the importance of Cajal's contributions to the study of the plasticity of the nervous system through his theoretical correlations between histological results and mental processes became obvious. In his first review published in Spanish (Cajal 1892), he reported his new concept on brain organization, first introducing the pyramidal neurons as the responsible for psychic functions, page 38: "**With some restrictions, it can be affirmed that in the animal kingdom psychic functions are bound to the presence of pyramidal cells (psychic cells) […] The number of elements of different category connected to one cell can be measured by the extension and the degree of differentiation reached by its protoplasmic arborizations [dendrites] […] Therefore, it can be estimated as likely that the psychic cell plays a wider and usefully activity when bigger are the number of its protoplasmic, somatic and collateral expansions, and more copious, longer and more branched are the collaterals coming out its cylinder—axis** [axon]."

A few lines below, on page 39, he reported his ideas about the morphological changes that should accompany the intellectual reasoning and the talent, resulting from what he called "cerebral gymnastics": "**These facts are: the notable increase in intellect observed among men dedicated to deep and continued mental exercise; and the coexistence of a notable talent and even of a true genius with a medium or smaller sized brain than those of normal weight and dimensions. In the first case, […] it can be supposed that cerebral gymnastics will lead to the development of protoplasmic processes and nervous collaterals beyond that normally observed, forcing the establishment of new and more extensive intracortical connections. […] In the second case, there is nothing to prevent us from accepting that certain brains, either because they inherit prior adaptations or through other causes, offer a notable development of all kind of collaterals in compensation for the smaller number of cells […]**".

Therefore, neurons can respond to functional activation like many other body tissues, especially muscle tissue. While strength training exercises in gymnastics

lead to muscle hypertrophy, mental exercise increases the intellect in parallel with the increase in the number of cortical synaptic connections.

It is important to notice that at the bottom of the page, Cajal made reference to Alexander Bain (he did not provide the complete reference), by saying: "**If understand is, as Bain says, to perceive similarities or differences between our ideas, the depth and breadth of judgment will be much larger, the greater the number of acquisitions or images will be used as material, and more extensive is the system of relationships that among them consents to establish the cellular substratum of the brain**." This sentence seems to sum up the ideas of Bain - expressed with Cajal terms- that the retention mechanism passes through growth in the number of cell junctions between nerve cells allowing the establishment of precise new neural groupings as previously discussed.

Indeed, 2 years later, at the occasion of his invitation by the Royal Society of London as speaker for the Croonian Lecture, Cajal (1894) delivered in French a new version of his ideas on the action of "cerebral gymnastics" on mental processes. He wrote pages 466–467: "**Cerebral gymnastics are not able of improving the brain organization by increasing the number of cells, because, it is known, that the nerve cells have lost after the embryonic period the property of proliferation; but […] mental exercise leads to a higher development of the protoplasmic extensions [dendrites] and of nervous [axonal] collaterals in the regions of the brain in use. In this way, associations already established among certain groups of cells would be significantly strengthen by means of the multiplication of the small terminal branchlets of the protoplasmic appendages and nervous collaterals; but, in addition, totally new intercellular connections could be established thanks to the neoformation of [axonal] collaterals and protoplasmic expansions** [dendrites]".

Cajal started by considering that even though his ideas were not original, the main difference now is that "cell junctions" take their real value, since they concern the morphological contacts (later named the synapses) between neurons. Therefore, after exposing his theoretical considerations, he finished: "**This anatomical-physiological hypothesis is not original in principle, because there are many physiologists and psychologists who have searched for the somatic characteristic of intelligence into the richness of cellular associations, but it has something new, it is based on positive facts on the brain structure and not on mere assumptions about the organization and the relationships of the nervous corpuscles.**"

In 1895, Cajal published in Spanish an interesting article entitled "Some conjectures about the anatomical mechanism of ideation, association and attention," which attempted to provide a mechanistic model of the phenomenon of consciousness, by explaining how the brain could pass from one stage of rest to another of activity. In this paper, Cajal first discussed the propositions of Hermann Rabl-Rückhard (1839–1905) who, in his publication on the study of a crustacean cerebral ganglion (1890), launched the hypothesis of amoeboid movements of neuronal processes (see Bawden 1900; DeFelipe 2006). This proposition was extended by Mathias Duval (1844–1907) to the nervous system of all living animals (1895). Accordingly, for Duval the amoeboid movement of neuronal processes would be able to cause their

retraction, with the separation of cell contacts, or conversely, their moving toward each other to restore the contacts. These movements could explain the inactive state induced by sleep, anesthetics, etc. (contact separation), while their approach, allowing the restoration of contacts, would be responsible for the states of brain activity (consciousness). In the last part of the paper, Cajal developed his adaptation of the Mathias Duval hypothesis. Based upon his failure, despite repetitive experiments, to establish direct correlations between the way of animal's sacrifice (chloroform, curare poisoning, bleeding) with the anatomy of neuronal processes, Cajal was forced to give up with this hypothesis. However, his observation on the cerebral cortex prompted him to adapt the hypothesis of amoeboid movements, from neuronal to astrocytic processes. He said: "**In contrast, our studies of the cerebral cortex have led us to suspect that, during mental work the morphology some glia cells varies. In the grey matter of the brain, neuroglial cells are sometimes shrunken, provided with short and stout appendages, some other times exhibit long, abundant expansions studded with infinite secondary and tertiary branchlets (cells of penniform expansions of Retzius). Between the retracted and relaxed states all transitions can be seen**".

During mental activity, the amoeboid movements of neuroglial processes would be enough to allow the withdrawal of thin astrocytic processes between axon terminals and postsynaptic elements, thereby restoring contacts that were disconnected during the mental rest. Opposite, the inactivity would raise the movement of thin astrocytic lamellae that would pull apart the pre- and postsynaptic elements, disconnecting this way inactive neuronal circuits. It is obvious that, even though a similar kind of plasticity linked with the withdrawal of astrocytic covering of supraoptic hypothalamic neurons has been reported (it takes place during the control and coordination of oxytocin and vasopressin release during special conditions such as lactation and dehydration; see in Hatton 1997), the mechanism conceived by Cajal has not been validated.

2.2.4 Synapses and Plasticity

After a period of decline between 1915 and 1940, during which the hypothesis of the synaptic location of learning was questioned (see Berlucchi and Buchtel 2009), it made a strong comeback in 1948 and 1949 with the publications from the neurophysiologist Jerzy Konorski and the psychologist Donald O. Hebb. In his important monograph, "Conditioned Reflexes and Neuron Organization," published in 1948, Konorski discussed the mechanisms underlying the behavioral changes induced by conditional reflexes. He foresaw that these mechanisms should be quite similar to those of the spinal reflexes, which are based upon the reorganization of synaptic connections. Donald O. Hebb (1949) in his book "The Organization of Behavior: A Neuropsychological Theory" proposed that the synaptic activity between two neurons strengthens when the neurons display simultaneous activity. Hebb reported his postulate as follows (Chap. 4, page 50): "**When an axon of cell A is near**

enough to excite a cell B and repeatedly and persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased." It is noteworthy to compare the similitude of this sentence of Hebb with the one of William James on page 566 of his book, written 59 years earlier (cited above).

The morphological correlate in this connectionist theory of learning is based upon the above-discussed principle developed by Tanzi, Lugaro, and Cajal of growth of synaptic connections (Berlucchi and Buchtel 2009), in Hebb's words (Chap. 4, page 50): "**When one cell repeatedly assists in firing another, the axon of the first cell develops synaptic knobs (or enlarges them if they already exist) in contact with the soma of the second cell (Soma refers to dendrites and body, or all of the cell except its axon)**."

Advances in physiology and morphology achieved in the second half of the twentieth century have focused the synapse as the seat of learning and memory. The discovery in 1973 by Tim Bliss and Terje Lømo of the long-term potentiation (LTP) of synaptic transmission in hippocampal neurons produced in response to a burst of high-frequency stimuli (tetanic stimulation), activating the perforant pathway to dentate granule cells in the dentate gyrus, the major synaptic pathways in the hippocampus, provided a direct proof that a gain in strength and effectiveness in synaptic transmission can result from a sustained high activity as that produced by experience. Furthermore, the fact that the hyperpolarization of a neuron is sufficient to prevent its LTP (Lin and Glanzman 1994) showed the validity of the Hebb's principle. Synaptic plasticity can be considered today as a major mechanism for memory formation.

2.3 Morphological Neuronal Plasticity: Remodeling of Synaptic Contacts and Anatomical Changes in Dendrites and Axonal Initial Segments (AIS) in Adult Non-lesioned Animals

As it is beyond the scope of this review, we will not discuss the integration of new neurons in adult circuits, although neurogenesis that occurs at least in rodent hippocampus and olfactory bulb is a beautiful example of morphological neuronal plasticity (see reviews in Deng et al. 2010 and Lepousez et al. 2013, respectively).

2.3.1 Dystrophic Axon Terminals and Dendritic Growth Cones

For years the morphologists have been exploring the CNS trying to find some clues supportive of the connectionist theory of learning. If the neuronal circuits would be in continuous remodeling, it should be possible to observe these morphological

changes at the synaptic level. The advent of electron microscopy paved the path toward the observation of a possible synaptic remodeling that should result from some kind of degenerative process for the removal of disused presynaptic axon terminals, followed by the formation of newly formed synaptic contacts (synapse formation/elimination and remodeling of axons and dendrites). Sotelo and Palay (1971), in their electron microscope study of the synaptology of the lateral vestibular neurons of apparently normal rats, reported the occurrence of enlarged nerve endings containing a variety of unusual structures already noticed years before in humans (infantile neuroaxonal dystrophy or Seitelberger's disease; Sandbank et al. 1970), as well as in experimental animals (Tellez and Terry 1968), and considered to be the consequence of pathological processes. The alterations seen mostly involve membranous organelles, almost exclusively appearing in axon terminals and preterminal fibers. Similar dystrophic axons have been also reported in the dorsal column nuclei (Hashimoto and Palay 1965), lateral vestibular nucleus of the cat (Mugnaini et al. 1967), hypothalamus (Dellman and Rodriguez 1970), superior cervical ganglion (Ceccarelli et al. 1971), deep cerebellar nuclei (Angaut and Sotelo 1973), and autonomic nerve endings (Townes-Anderson and Raviola 1978). It is important to underline that some of these dystrophic axons were in continuity through a thin neck with a small axon terminal of normal appearance, as if the small extension was originated from the large abnormal terminal by a budding of process (Fig. 25 of Sotelo and Palay 1971). Sotelo and Palay proposed that the dystrophic axons were degenerating, whereas the budding off would represent the regenerative counterpart.

Together with this axonic remodeling, Sotelo and Palay (1968) also reported the presence of large dendritic varicosities at the tip of some dendritic branches, which received synaptic inputs from nearby axon terminals. These dendritic profiles were crowded by a very large population of mitochondria and by glycogen particles, together with customary organelles. These profiles, more abundant in the dorsal than in the ventral regions of the nucleus, are not the attribute of the lateral vestibular nucleus and have been encountered in other regions such as the cerebellar nuclei (Sotelo and Angaut 1973), superior cervical ganglion (Ceccarelli et al. 1971), and autonomic nerve endings (Townes-Anderson and Raviola 1978). Their location, connection with the parent dendrite, and, mainly, correlation with Golgi-impregnated preparations (Sotelo and Angaut 1973) have allowed to conclude that these profiles might represent at least the distal segment of dendrites, if not their actual tips, and were considered as growth cones of a growing or regenerating nerve cell dendrite. The hypothetical occurrence of axon terminals in different stages in a cycle of degeneration and regeneration of nerve endings in the normal animal, together with the possibility of dendritic growth, was taken as indirect proof of a continuous remodeling of synaptic connections. This way, axons and dendrites might engage in such a process, reflecting the continued experience of the animal, making and discarding interneuronal connections by the activity of both pre- and postsynaptic elements. Although this hypothetical interpretation was tempting to investigators interested in finding a morphological substrate for the process of learning, these

static results were insufficient to provide a morphological base to the connectionist hypothesis of learning and were forced to oblivion.

2.3.2 LTP and Dendritic Spines

One of the essential paradoxes to be handled by the brain is to preserve the right balance between two seemingly contradictory conditions: the stability of the already established neuronal circuits, required for preserving behaviors and long-term memory, and the constant structural changes needed for the synaptic plasticity underlying learning. Therefore, the morphological changes in normal learning conditions should be limited but should occur. The most broadly studied synaptic changes occur in excitatory axospinous synapses and are mainly characterized by the addition or removal of a small fraction of their postsynaptic dendritic spines, causing minor quantitative changes.

Cajal (1888), in his first study of the cerebellar cortex impregnated by the Golgi method, reported the existence of spines, as peculiar tiny excrescences emerging along the distal compartment of Purkinje cell dendrites. After long and tedious discussions to determine if the spines were only the result of artifactual precipitation of silver over the neuronal surface or belonged to true protrusions designed to increase the receptive surface area of the dendritic tree, its reality was firmly established by Cajal (1896) when he was able to replicate their appearance with a vital dye—the methylene blue method—in cerebral cortical neurons. George Gray (1959), 63 years later, corroborated the existence of dendritic spines during his electron microscope study of the rat visual cortex. Gray added two important attributes to spines: they almost systematically establish asymmetric synaptic contacts (a morphological marker of excitatory synapses) and have a specific organelle—the spine apparatus—of unknown function.

From the discovery of LTP (see above), neuroscientists have tried to correlate the long-term potentiated synapses with their presumptive morphological counterparts. Since the targets for the axons of the perforant path originated in the entorhinal cortex are the spines of the granule cell dendrites of the dentate gyrus, these dendritic spines have been for the last 37 years the matter of numberless quantifications. The analysis of several parameters tempted to provide a solid structural support to the observed functional modifications. So, changes in the preexisting synapses concerning either the length and width of the spines or of their active zones, the shape and size of postsynaptic differentiations, or changes in the number of synapses in potentiated compared to non-potentiated hippocampi were addressed mostly with electron microscopy (Van Harreveld and Fifkova 1975; Fifkova and Van Harreveld 1977; Fifková and Anderson 1981; Geinisman et al. 1989; Desmond and Levy 1990; Trommald et al. 1996; Harris et al. 2003; for a more detailed bibliography see in Bailey and Kandel 1993). After laborious quantifications, despite the solidity of some of the positive results, their inconsistency did not permit any definitive conclusion that LTP, prolonged for minutes and even hours, was able to provoke

reproducible structural alterations of axospinous synapses on dentate granule cells. The problem was solved later on with the advancement of new imaging techniques (see below).

2.3.3 *Recent Progress in Live Cell Imaging Has Finally Demonstrated the Occurrence of Synaptic Remodeling: Formation and Pruning of Spines*

The progresses in physical sciences and informatics have generated new tools for the study of the organization of the brain and the ability to examine the dynamic aspects of the neuron. For instance, the development of computer-assisted image analysis has promoted new kinds of quantitative studies, such as three-dimensional morphology of Golgi-impregnated neurons with their dendritic and axonic fields or 3-D reconstruction of neuronal compartments from electron micrographs. The development of confocal laser scanning microscopy (CLSM) has provided an enhanced resolution, compared to classical fluorescence microscopy, and has allowed the 3-D reconstruction of thick sections. Advances in laser technology during the last 10 years have permitted the development of a new type of CLSM that, keeping all the advantages of the confocal microscopy (ability to penetrate scattering tissues), have added the advantage of greatly reducing phototoxicity and photobleaching. This is the two-photon laser scanning microscopy (TPLSM), where fluorescence excitation above or below the plane of study is practically nonexistent. Thus, it can be used to study thick brain slices in organotypic culture or even neuronal compartments located several hundreds of micrometers deep in the living brain. These recent improvements have opened a new epoch of neuromorphology, because the observations in living tissues or animals have added the dimension of time to this discipline. In fact, under favorable conditions, it is possible, after repeated examinations, to analyze the morphology of a neuron, its dendritic and axonal fields, as well as its dendritic spines over prolonged periods, allowing the detection of presumptive morphological changes.

Engert and Bonhoeffer (1999), by using local superfusion technique together with two-photon imaging, were pioneers to directly observe in living hippocampal slices that the induction of LTP in synapses of CA1 region was able to initiate neospinogenesis, whereas in non-stimulated regions of the same dendrite or, more interesting, in slices where long-term potentiation was blocked, spinogenesis did not occur. A similar high dynamic of dendritic spines was corroborated by numerous investigators (see, for instance, Leuner and Shors 2004; Kasai et al. 2010 for a review). These first data, reporting intense spine remodeling in hippocampal and cortical neurons, were later on reevaluated because they were obtained from young, still somewhat immature, neurons, and it is likely that spine lifetimes vary greatly. Therefore, even though neosynaptogenesis and synaptic pruning take place throughout the life span, after the critical period, when the brains are reaching maturity,

spine dynamics slow down and become stable. This stability has been confirmed in many studies and with different experimental paradigms (Grutzendler et al. 2002, see in Bhatt et al. 2009). Moreover, age difference was quantified by Zuo et al. (2005), who working on spines on the apical dendrites of layer 5 pyramidal neurons of the barrel, motor, and frontal cortices reported in observations conducted over 2 weeks that while in 1-month-old mice 13–20 % of spines are eliminated and 5–8 % formed, in 4–6-month-old adult mice, the remodeling was much lesser since in 2 weeks only 3–5 % of the spines were eliminated and 5–8 % formed. The results were more impressive if the observation period is prolonged over 18 months, only 26 % of spines were eliminated and 19 % formed in the adult barrel cortex, testifying for the stability of spines in adult brains.

Finally, to close this section on dendritic spines, we believe that it is worthy to mention the recent paper by Lai et al. (2012). These authors have investigated, by transcranial two-photon microscopy, the structural modifications of neuronal circuits in the mouse frontal association cortex by analyzing changes in dendritic spines of layer-V pyramidal neurons during fear learning and extinction. These authors arrived to an important conclusion: fear conditioning, extinction, and reconditioning lead to opposing changes at the level of individual synapses because reconditioning after conditioning and extinction eliminates those newly formed spines stabilized by extinction. Therefore, stabilization appears as an essential key required for memory storage, and the persistence of new spines can be considered as engrams in learning.

2.3.4 Changes in Dendritic Spines Are Correlated with Changes in Presynaptic Axon Terminals. Examples from the Hippocampus

In a recently published paper by the group of Michael Frostcher (Zhao et al. 2012), structural changes of mossy fibers and their postsynaptic counterpart have been associated with chemically induced LTP in slice cultures, taken from newborn mouse hippocampi and kept for 14 days in vitro. Ten minutes before fixation, the cultures were treated with tetraethylammonium (TEA), a blocker of potassium channels, to induce LTP. The interest of this work is that in order to avoid all possible artifacts consecutive to the chemical fixation of the biological material, the authors have used high-pressure freezing physical fixation. In wild-type mouse hippocampi, the length of the membrane of the mossy fiber boutons was increased and associated with an increase in the number of active zones and of postsynaptic spines.

Pico Caroni and collaborators (Galimberti et al. 2006) analyzed the synaptic remodeling between granule cell axons and the dendritic spines of pyramidal cells in the hippocampal CA3 region. In control adult mice, each granule cell axon established 10–15 complex synaptic arrangements—the mossy fiber varicosities—that are changeable throughout life. More importantly, the quasi constant remodeling of

these complex synapses was greatly enhanced just by keeping the mice in an enriched environment. Comparable results were obtained in organotypic cultures of the hippocampus, where it was possible to demonstrate that the generation and maintenance of the plastic changes required spiking activity in the slices and transmitter release from mGluR2-sensitive receptors, because blocking this release with an mGluR2 agonist reversed the structural plastic changes. Functionally speaking, the plasticity changes by increasing the size of active zones in presynaptic membrane that allows for a stronger excitatory response in the postsynaptic neurons, which could lead to an increased complexity of the mossy fiber circuits, the presumptive substrate for increased hippocampal learning. More recently, Pico Caroni and collaborators (Ruediger et al. 2011) were able to show in adult mice after one trial and incremental learning that mossy fibers in the hippocampus and cerebellum present long-lasting and reversible increases in the numbers of filopodial synapses onto fast-spiking interneurons that trigger feed-forward inhibition. From different types of experiments, the authors showed a causal relationship between the morphological data (increase in number of synapses) and the precision of learning and memory. Thus, they proposed that feed-forward inhibition growth at hippocampal mossy fibers fitted with the precision of hippocampus-dependent memories. The special interest of this last paper is that, although the majority of the work related to the structural plasticity of axospinous synapses has been focused on glutamatergic excitatory transmission, Ruediger et al. (2011) have shed light on the important role that local inhibitory circuits have in the acquisition of learning and memory.

2.3.5 Changes in Dendritic Tree Morphology

The group directed by Dale Purves was a pioneer in the analysis of dendritic trees in in vivo specimens. Indeed, with Robert Hardley and James Voyvodic (Purves et al. 1986), they described the methodology to visualize, over intervals from 1 day to up to 3 months, the same neuron in repetitive experiments. These early studies were performed with peripheral neurons, those of the superior cervical ganglion of young adult mice, by injection of nontoxic vital fluorescent dyes and laser scanning confocal microscopy. The obtained results corroborated the working hypothesis, the occurrence of extensive remodeling in dendritic trees, the postsynaptic elements in the sympathetic ganglion. The important changes consisted in growth of some of the branches, with retraction or even disappearance of others, and the de novo formation of still other dendrites. These results provided the idea for a relatively extensive dendritic remodeling in adult sympathetic neurons that does not correspond to the actual concept we have of the neuronal stability. Here again, the age of the animals could be responsible of the early results.

A few years later, Dale Purves started another important study directed to determine whether central neurons, in this case those involved in the formation of olfactory bulb glomeruli, were continuously changing both during development and in adulthood. For that purpose, newborn, juvenile, and adult mice were analyzed over

intervals of several hours to several weeks (LaMantia et al. 1992). The results emphasize that while in newborn and juvenile mice the development is not finished, and new glomeruli are added progressively, in adult mice there is no longer addition of new glomeruli, and the neuronal population involved in glomerular formation remains stable, as it was the case for dendritic spines (see above). In conclusion, despite the impressive stability of dendritic processes and spines in adult brains, there is a slow pace remodeling throughout the whole life of the animals.

2.3.6 Changes in Length and Position of the AIS

The last neuronal region to be characterized with the electron microscope was the axon initial segment (AIS). It was only identified in 1968 (Palay et al. 1968), as a specialized and important region, which emerges from the axon hillock and separates the axonal from the somato-dendritc compartment, acting as selective tight molecular filter for the axoplasmic transport (Song et al. 2009). AIS is therefore the command region for axonal identity and responsible for neuronal polarization. It has been morphologically identified by the occurrence of three specific ultrastructural features: (a) fascicles of microtubules, (b) sparsely distributed clusters of ribosomes, and (c) a dense layer of finely granular material undercoating the plasma membrane. A similar undercoating occurs beneath the plasma membrane of myelinated axons at the nodes of Ranvier (Andres 1965, Fig. 5). This anatomical region is where the action potentials are initiated (Coombs et al. 1957) and is assumed to possess a membrane provided with a high density of voltage-gated sodium (Na 1) channels (Kole et al. 2008).

During the last 6 years, it has been established that the AISs, with their strategic location and important functions, are not static in structure but also subjected to plasticity. These studies started with the electrophysiological analysis of the auditory brainstem neurons in the newly hatched chicken (between P2 and P5) (Kuba et al. 2006). In a recent review, Hiroshi Kuba (2012) summarized his work explaining that in the nucleus magnocellularis (involved in a relay of timing information) the length of the AIS changes in relationship with sound frequency (increases with decreasing frequencies) to accommodate frequency-specific variations in synaptic inputs. In the nucleus laminaris (involved in integrating time from sounds received from both ears for their localization) the length and the location of the AISs vary depending on sound frequency; they are shorter and more remote for higher frequency. Juan Burrone and Matthew Grubb have corroborated and expanded the study of AIS plasticity (Grubb and Burrone 2010). They used dissociated cultures of hippocampal neurons taken from E18 rat fetuses. By increasing the levels of activity, these investigators were able to change the precise location of the AISs of the stimulated excitatory neurons. When the neurons, for example, were kept in vitro for 10–14 days in high extracellular potassium concentration (15 mM) to chronically depolarize them or in neurons transfected with the light-activated cation channel channelrhodopsin-2 and photostimulated, in these situations the molecular

components of the AIS shifted up to 17 μm away from the neuronal perikaryon. These effects were reversible, and when the excitatory neurons were replaced in a 3.5 mM potassium medium, the AIS is relocated at its original place. Therefore, long-term changes in electrical activity can cause important changes in AIS location. Due to the essential role of AIS in the firing rate of the neurons and the correlation between its location and the thresholds for action potential spiking, shifting positions of the AIS appear as a mechanism for homeostatic plasticity for cell autonomous modulation of neuronal excitability (Grubb and Burrone 2010; Kuba et al. 2010).

2.3.7 The Influence of the Environment as a Motor Controlling the Number and the Plasticity of Synapses

One of the postulated principles of a plastic brain is that as a result of its constant exchanges with the exterior world, it should be constantly malleable by external causes. The principal causes encountered during animal life will be either changes in its physical environment or responses to injuries of its CNS. A fortuitous discovery of Mark Rosenzweig and collaborators was to observe modifications of brain circuitry through experience due to changes in the environment encountered by caged rats. As reported by Rosenzweig in a recent (2007) historical review, it was during the late 1950s and beginning of the 1960s that in collaboration with a neurochemist (Edwards Bennett) using acetylcholinesterase (AChE) activity as a parameter considered to be stable that they showed this value was partially dependent on the animal's experience. The experienced animals as a consequence of their enriched environment were those kept in company with at least 10–12 other rats, in large cages provided with 5–6 objects to play, explore, and climb upon (e.g., wheels, ladders, and small mazes that were changed in location or replaced by new ones two to three times a week); they were considered rats raised in enriched environment. Control animals were raised in standard laboratory conditions, and the third group consisted of partially sensory-deprived rats either raised in isolation or maintained in groups up to three, in small empty cages. In rats kept in enriched environment, an augmentation in AChE activity was correlated with accrual depth and weight of the cerebral cortex, together with a decrease, per examined field, of neurons, glia, and capillaries suggestive of increased dendritic branching (Bennett et al. 1964; Diamond et al. 1964). Therefore, social interactions together with increased exercise were able to boost learning and memory in the subjected rats, as demonstrated with the behavioral tests used, and paralleled the augmentation of chemical and morphological parameters of the cerebral cortices of the experienced rats. More interesting, the behavioral, anatomical, and chemical changes do not only occur at weaning but also in adult full-grown rats after periods as long as 30 days in the enriched environment.

From the viewpoint of our chapter on structural plasticity, the work of William Greenough and his collaborators is also of great interest. While Marian Diamond

was measuring parameters linked to the size of the cortex and its cellular elements, such as cortical thickness, diameter of neurons and of their nuclei, number of glial cells, and capillaries, as we referred to above, William Greenough and collaborators were more interested in parameters linked with synaptic connections such as dendritic dimensions and branching and dendritic spines, not only in the visual cortex where the encountered differences between control and experienced animals were the largest (Greenough and Volkmar 1973) but also in the hippocampus and entorhinal cortex (Fiala et al. 1978), basal ganglia (Comery et al. 1995, 1996), and cerebellar cortex (Greenough et al. 1986). The dendritic fields of the neurons of rats kept in enriched environments exceed by 20 % the size of those in control animals (Greenough and Volkmar 1973; Volkmar and Greenough 1972). Working on the adult rat visual cortex kept in the enriched environment (Juraska et al. 1980), they were able to show on Golgi-Cox-stained neurons that the size increase of the dendritic trees of the experienced rats was selective for layer IV stellate cells. In these neurons there was an increase in the number of first-order stem dendrites as well as in the fifth-order distal branches. Structural changes were, therefore, obtained as a result of life for 12 weeks in the enriched environment, but the change was not general but targeted to specific neurons. A few years later, the quantification was extended to the density of synaptic boutons (Turner and Greenough 1985) and the size of some synaptic components in layer IV, mainly the length of the synaptic complexes (Sirevaag and Greenough 1985). Greenough's group has also shown that the resulting plasticity occurring from exposure to the enriched environment is not age dependent, since it can occur even in aging rats, with a similar increase in synapse number and dendritic branching (Greenough et al. 1986).

In addition, the work of Greenough has also corroborated and expanded on the results of the Rosenzweig team concerning the fact that not only neurons are able to change due to experience, since the enriched environment is also able to affect practically all classes of nonneuronal elements present in the CNS, and result in angiogenesis, increased myelination, and astrocytic hypertrophy with *ensheathment* of synapses (see in Markham and Greenough 2004).

The interactions between the environment and brain plasticity are very complex and far to be solved. Recently, the group of Gerd Kempermann demonstrated that factors unfolding or emerging during development contribute to individual differences in structural brain plasticity and behavior (Freund et al. 2013). They conclude their article by writing the ways in which living our lives makes us who we are.

2.4 Morphological Neuronal Plasticity Consecutive to Neuronal Lesions

We have just reviewed the evidence that despite the apparent stability of the brain circuits, the adult brain is characterized by constant synaptic remodeling, often accompanied with slight modifications in the size and branching of dendritic trees

and of the somatic volume. Now, we want to discuss another kind of structural plasticity, the one taking place as a consequence of lesions that provoke the partial deafferentation of postsynaptic neurons.

It has been known for many centuries that persons suffering from cranial trauma or cerebrovascular problems, although they never recuperate their normal behavior, are capable of progressive improvement during the first months following the instauration of the neurological deficits. Since, contrary to what happens in the peripheral nerves that are able to regenerate, in the CNS, axonal regeneration is abortive (Cajal 1913, 1928), therefore, there must be other types of reparative processes responsible for the observed improvement. One of these possible mechanisms is likely the disposition showed by injured axons to grow locally, so-called axonal sprouting. Not only lesioned axons, if their neurons of origin do not degenerate (retrograde degeneration), are able to sprout, as reported already from the beginning of the twentieth century by Bielschowsky, Nageotte, and Marinesco (see Stahnisch 2003), but also intact nearby axons.

An excellent description of the after stroke improvement was provided by Alf Brodal (Professor of Anatomy at the Medical Faculty in Oslo) of his self observations after a left-sided hemiparesis without hemianopsia resulting from an embolic lesion of the posterior part of the right internal capsule (Brodal 1973). Alf Brodal finished his description saying: "**It seems that some of the recovery after a brain lesion may be explained by reinnervation from remaining fibres of synaptic sites which have been denuded as a consequence of the interruption and degeneration of afferent fibres**." It is this process, first reported by Geoffrey Raisman (1969), that was named "terminal sprouting or terminal plasticity" (Sotelo 1975).

2.4.1 Terminal Sprouting

Work carried out with silver impregnation methods showed that the destruction of a source of afferent fibers to a central nucleus causes a compensatory sprouting of the remaining afferents synapsing on the partially denervated neurons (Liu and Chambers 1958). This compensatory process might occur either from collateral sprouting of remote fibers or from a terminal sprouting of nearby fibers. Geoffrey Raisman (1969) was able to settle this dilemma owing to the anatomical arrangement of the afferent fibers entering the septal nuclei. Indeed, these nuclei receive their afferents fibers from two main external sources, the medial forebrain bundle (MFB) and the fimbria. Ultrastructural studies using anterograde degeneration after specific lesions in either of these two kinds of afferent axons revealed that fibers from the MFB established synaptic connections on both dendrites and perikarya, whereas synapses from fimbrial axons were restricted to dendrites. The important observation was that several weeks after destroying one of these afferent fibers, the remaining afferents go beyond their normal boundaries and occupied postlesionally released postsynaptic differentiations (PSDs). Thus, the transection of the MFB induced the formation of axosomatic synapses by fimbrial axons that, according

Raisman (1969), had moved from their locations by forming short terminal sprouts that ended by occupying the PSDs vacated after the MFB lesion. These neoformed terminals degenerated after a later destruction of the fimbria, corroborating their initial origin.

A prerequisite for this type of short-length axonal sprouting is that denervated postsynaptic membranes must withhold part of their molecular machinery (receptors and associated transmembrane and cytoplasmic molecules) when they are released from their presynaptic partners so that they would be able to attract intact synaptic boutons from their surroundings. Morphological evidence in favor of the possibility that some PSDs can retain their anatomical structure despite having lost their presynaptic elements was provided by the observation of free PSDs in somatic and dendritic membranes of partially deafferented neurons. Indeed, a frequent observation among electron microscopists trying to identify the origin of axon terminals by anterograde degeneration was the permanence of some normal-looking PSDs after the lesion, for instance, in the avian optic tectum after sectioning of the contralateral optic nerve (Gray and Hamlyn 1962) or after cutting the presynaptic fibers in the frog sympathetic ganglion (Hunt and Nelson 1965), where even the more complex PSDs provided with a subsynaptic bar can persist for over 12 days, strongly suggesting that these structures are not intimately related to the functional integrity of the axon terminals (Sotelo 1968). Examples of persistence of postlesional vacated PSDs in the CNS are numerous, such as the prepyriform cortex (Westrum 1969), the inferior colliculus (Lund 1969), the olfactory bulb (Pinching 1969), or the dorsal column nuclei, where normal-looking, vacated PSDs lasted for at least 6 months (Rustioni and Sotelo 1974).

The attraction exerted by the vacated PSDs and the consequent reinnervation cause the loss of one of the essential qualities of synaptic connections, their specificity, and, therefore, we termed the newly formed synapses “heterologous.” The problem with the presumptive loss of specificity was analyzed in two different central nuclei: the anterior ventral cochlear and the dorsal column nuclei, particularly in the nucleus gracilis (Gentschev and Sotelo 1973; Rustioni and Sotelo 1974). In the auditory nucleus, the primary sensory fibers ended as large axon terminals containing rounded vesicles, establishing asymmetrical synapses that correspond to excitatory terminals of the cochlear nerve. The intact axon terminals were smaller, contained flattened vesicles, and established symmetrical synapses in close proximity to the larger primary sensory terminals. The smaller terminals belonged to other systems of fibers (higher auditory centers, mainly the superior olivary complex with inhibitory action) that moved by a sliding process to reinnervate the vacated PSDs. It can be therefore suggested that this type of synaptic adjustment, with loss of the specificity of the nervous organization, could aggravate the unbalanced state of the primary deafferented neuron. In fact, the loss of excitatory inputs seems to generate an increase in inhibitory inputs on the anterior ventral cochlear neurons. As far as the morphological data can be interpreted, the persistence of free PSDs could therefore be harmful to the function of the neuron, if the newly formed synaptic contacts are functional. The situation is quite similar in the nucleus gracilis, although in this system the large primary sensory axon terminals systematically, and not only

occasionally, receive serial synapses from the smaller boutons forming complex synaptic arrangements similar to those encountered in the glomeruli of the substantia gelatinosa of the spinal cord, where the axon terminals of the primary sensory fiber are postsynaptic to the surrounding flattened vesicle containing boutons. The transection of the dorsal roots induced the degeneration of the large primary sensory terminals and the sliding of their presynaptic boutons until their reoccupation of the vacated PSDs. The initial stage of the "reoccupation" of vacated PSDs is characterized by a partial apposition of the bouton-shaped terminal to the free postsynaptic sites; in some instances, the broken presynaptic membrane or a thin layer of astrocytic cytoplasm can be interposed between the deafferented zone and the invading terminal. Finally, the invading terminal develops a new presynaptic vesicular grid facing the deafferented PSD. This last stage is more easily identifiable when the two presynaptic terminals share the vacated PSD, both of them establishing active zones on either half of the PSD.

The fact that only a small fraction of the vacated PSDs persist and succeed in being reinnervated lets us assume the occurrence of a protective mechanism to avoid the harmful effect that, as discussed above, could provoke the formation of the heterologous synapses by unbalancing the required equilibrium between excitatory and inhibitory inputs. Gentschev and Sotelo (1973) reported that in the anterior ventral cochlear nucleus, the sequestration of vacated PSDs could be part of this protective mechanism. In fact, the most common fate of the vacated PSDs is their disappearance by engulfment into the postsynaptic cytoplasm by way of formation of small bags which contain some remnants of presynaptic organelles and which invaginate the surface of the postsynaptic element. From days 5 to 9 these pre- and postsynaptic remnants enter into the postsynaptic cytoplasm by a large pinocytosis, where the remnants of the synaptic complexes become totally engulfed in the neural cytoplasm until their disappearance probably by lysis through the lysosomal system. In the nucleus gracilis a similar mechanism to get rid of vacated PSDs was also disclosed (Rustioni and Sotelo 1974). This last result indicates that membrane sequestration is not specific of the deafferented anterior ventral cochlear nucleus and may be considered as a protective mechanism that exists in other nuclei to regulate the rate of axonal remodeling by terminal sprouting.

2.4.2 Collateral Sprouting from Axotomized and Non-axotomized Axons: Examples After Spinal Cord Injury

In the context of reparative research after traumatic injury, neuroscientists have been impregnated by the idea that axons do not regenerate within the mammalian adult CNS whereas they can in the PNS (Cajal 1928). Thus, until the beginning of this century, most studies dedicated to promote functional recovery after traumatic injury have been focused on how to promote axon regeneration in the adult mammalian CNS (Thuret et al. 2006). It is largely understood that neurons fail to

regenerate their axons in the CNS for two reasons: (1) the lack of growth-permissive molecules and/or the presence of growth-inhibitory molecules in the environment of the severed neurons and (2) their inability to reactivate the genetic programs for axon growth (Dusart et al. 2005; Yiu and He 2006; Moore and Goldberg 2011). Thus, in the last decades, there has been a major effort to enhance spinal cord axonal regeneration through a variety of techniques including neutralization of neurite inhibition, administration of neurotrophic factors, and transplantation of a variety of cell types (see for reviews Filbin 2003; Rhodes and Fawcett 2004; Schwab 2004; Silver and Miller 2004; Thuret et al. 2006; Yiu and He 2006). Very surprisingly in many of these experiments, despite an effect on functional recovery, the number of axons that have been observed to regenerate is always low (between 1 % and 10 % when estimated) whatever the experimental approach (sciatic nerve, Aguayo 1985; ensheathing cells, Li et al. 1997; Nogo neutralization Schnell and Schwab 1993; and combination of treatments, Lu et al. 2004). Thus, functional recovery occurs in the presence of a very low number of regenerating axons. For instance, the group of Geoffrey Raisman has estimated that the presence of 1 % of the corticospinal axons beyond the lesion site is sufficient to produce significant functional recovery (Li et al. 1997). One difficulty to interpret these apparently controversial experimental results obtained after spinal cord injury is to determine the anatomical and functional bases of the recorded recovery. Indeed, important processes other than axon regeneration occur after spinal cord injury, either spontaneously or as a reaction to the applied treatment. For example, around the lesion site, demyelination of ascending and descending uninjured fibers has been observed, and over long periods after the lesion, spontaneous remyelination of these non-axotomized fibers by oligodendrocyte progenitors or by Schwann cells that invade the lesion area can contribute to the functional recovery by restoring conduction (Totoiu and Keirstead 2005).

Important components of functional recovery in the absence of regeneration of lesioned axons are the reorganization of circuits that have been spared by the lesion and the formation of new circuits through collateral sprouting of lesioned and non-lesioned axons (for review see Raineteau and Schwab 2001). The group of Martin Schwab has described the spontaneous formation of new circuits through collateralization of severed axons after spinal cord injury (Bareyre et al. 2004). Indeed, after incomplete spinal cord injury in rats, transected hindlimb corticospinal axons form collaterals into the cervical gray matter that contact short and long propriospinal axons. After 12 weeks, the contacts with the short propriospinal axons are no longer detected, whereas the ones with the long propriospinal axons that bridge the lesion site are maintained (Bareyre et al. 2004). Furthermore, treatment after rodent or primate spinal cord injury with Nogo-A neutralizing antibody (Nogo being one of the major myelin axon growth inhibitors present in the CNS) promoted not only axon regeneration of damaged fibers but also sprouting from intact fibers (Liebscher et al. 2005; Freund et al. 2006; Maier et al. 2009). Indeed, when a unilateral section of the corticospinal tract (CST) was performed at the level of the brainstem, axons grew from the contralateral non-lesioned CST and innervated the denervated side of the spinal cord in rats treated with a Nogo-A neutralizing antibody (Thallmair et al.

1998). Treatment after different types of injury with the bacterial enzyme chondroitinase ABC (ChABC) digesting the glycosaminoglycan chains on chondroitin sulfate proteoglycans (another major class of inhibitory molecules in the adult CNS) restores plasticity in adult CNS (Galtrey and Fawcett 2007; Carulli et al. 2010). Furthermore, chondroitinase ABC promotes compensatory sprouting of the intact corticospinal tract and recovery of forelimb function following unilateral pyramidotomy in adult mice (Starkey et al. 2012). Last, it has been recently described that the combination treatment caused a further increased of both axon regeneration and formation of collaterals from unsectioned axons (Zhao et al. 2013).

Although further works are necessary to unravel the importance of collateral formation in behavior recovery, it is an import point to consider when therapeutic treatments are proposed for spinal cord injury recovery.

2.5 Repair of Adult Central Nervous System by the Way of Cell Transplantations

2.5.1 Introduction

Despite the presence of neural stem cells in the CNS, from analysis of numerous genetic, traumatic, vascular, or experimental lesions, it is a certainty that a complete spontaneous restoration of the destroyed neurons and circuits along with disappearance of all neurological symptoms is not possible. For this reason, neuroscientists have been trying for over a century to bypass this limit by cell therapy, transplanting neurons—at the early times of transplantation—and stem cells during the last 20 years (immortalized cell lines, Renfranz et al. 1991, Snyder et al. 1992; adult neural stem cells, Arvidsson et al. 2002; and embryonic stem cells, McDonald et al. 1999, Björklund et al. 2002) to substitute for the missing neurons and palliate the caused impairments.

In the earliest experimental trials (Thompson 1890; Saltykow 1905; Del Conte 1907), the donor material was taken from postnatal animals, and a few days after the operations, all transplanted neurons had died, leaving only the nonneuronal supporting structures. Ranson (1909, 1914) reported the first successful transplantation of the dorsal root ganglia to the brain, even if the ganglia were taken from rats of at least 1 month of age. In his temporal longitudinal studies, Ranson showed that among the surviving cells the form of the spinal ganglion cell is not stable and fixed but is capable of undergoing marked alteration in a short space of time, similarly to what Nageotte (1906) described for pathological conditions of the human dorsal root ganglion cells in “tabes dorsalis” or in experimental animals after transplantation into the rabbit ear parenchyma (Nageotte 1907) in which the unipolar ganglion cells are transformed into multipolar irritated cells.

2.5.2 Transplantation of Embryonic Nervous Tissue into the Adult Brain

The real history of neural cell transplantation into the adult mammalian brain with a therapeutic aim started last century, at the middle of the 1970s (Stenevi et al. 1976; Björklund et al. 1976). The pioneering work of Gopal Das (Das and Altman 1971) and the work of Anders Björklund and Ulf Stenevi (1976, 1979) have defined the main conditions for the survival of transplanted cells (mainly the age of the donor cells that must be taken at the end of the proliferative period of the neuronal population to replace). Even though the results obtained with this approach (the grafting of embryonic solid pieces or isolated cell suspensions into the lesioned adult brain) have not been as successful as expected, for our purpose of the study of structural plasticity, they have been extremely useful. Indeed, this tool has allowed us to appraise the impact that embryonic neural cells can exert on mature neurons and vice versa. In normal conditions this confrontation of young neurons in a mature tissue only happens for the few neurons originating from the neural stem cells in the subventricular zone and the hippocampus. The main question to be solved was as follows: are embryonic neurons able to properly interact with adult neurons leading to their normal development and subsequent integration into the deficient adult neural circuits? But before addressing this question, let us make a short digression to discuss the normal way central neural circuits are organized in the mammalian brain.

2.5.2.1 Global Versus Point-to-Point Systems

Two organizational levels characterize the neuronal arrangement in the central nervous system. The vast majority of the central circuits, those involved in the transfer of specific patterned information such as sensory and motor systems, are organized following a plan of precise cell-to-cell connections that we define as "point-to-point" systems (Sotelo and Alvarado-Mallart 1986) and are based on the absolute specificity of synaptic contacts established between the constitutive neurons. Superimposed on this kind of basic circuits, there are some others considered to be modulatory or regulatory systems (concerning the peptidergic and particularly the monoaminergic system: noradrenergic, dopaminergic, serotonergic, and cholinergic) and that we coined "global systems" owing to their diffuse projection throughout the CNS (Sotelo and Alvarado-Mallart 1986). In addition, these "global systems" establish few morphologically detectable synaptic connections. For instance, in the serotonergic system of the cerebellar cortex, less than 9 % of its varicosities were involved in the establishment of "synaptic complexes"—the morphological correlate of the chemically transmitting synapses—and were considered as non-junctional terminals (Beaudet and Sotelo 1981) releasing their neurotransmitter in a paracrine fashion, as described for other regions of the CNS (Mobley and Greengard 1985). The neurotransmitter diffused through the extracellular space over large distances,

acting on those neurons equipped with adequate receptors and located within its precise diffusional volume. Our "point-to-point" and "global" systems were renamed a few months later: "wiring transmission" and "volume transmission," respectively (Agnati et al. 1986).

The interest of this distinction is that the grafted neurons in "global systems" exert their functional effects by paracrine release involving diffuse secretion of deficient neurochemicals in appropriate terminal areas within the host brain, without the necessity of the formation of specific synaptic connections between grafted and host neurons. By contrast, functional recovery following transplantation in "point-to-point" systems requires a much more precise morphological reconstruction involving precise regulation of the migration and the formation not only of afferent but mainly of efferent synaptic connections of grafted neurons, in such a way that the transplanted cells replace the missing cells. It is probably by this anatomical difference that transplantation has been much more broadly used in "global" than in "point-to-point" systems because the aims to fill are less constrictive in the former than in the latter. For instance, in animal models of Parkinson disease, grafting experiments have been over 10 times more numerous than in those of cerebellar ataxias.

The first indication that grafts can exert some positive effects in the improvement of the neurological symptoms originated in neurotoxic, traumatic, or genetic animal models of neurodegenerative diseases was obtained in experiments conducted in 1979 by Björklund and Stenevi. In these experiments, the "global system" constituted by the nigrostriatal pathway of adult rats was destroyed by the neurotoxin 6OHDA injected stereotaxically. This lesion reproduced symptoms corresponding to the clinical signs of the Parkinson disease. Björklund and Stenevi (1979) were able to show that grafts of DA-producing neural tissue (mesencephalic substantia nigra anlage) taken from rat fetuses, implanted into the striatum, could reduce some of the deficits caused by the 6OHDA intraparenchymal injection. For us, it is not casual that the history of modern neural cell transplantation started with the cell substitution in a "global system" because, as stated before, the possibility of functional replacement does not require a rebuilding of a mirror image of the destroyed neural circuit.

2.5.2.2 Transplantation in Murine Cerebellar Mutants as a Model of Grafting in "Point-to-Point" Systems

The relative simplicity and perfect geometrical arrangement of the cerebellar cortex, with few neuronal populations and inputs from various cell groups making synaptic connections at the precise site of the dendritic tree of the PCs—the unique output from the cortex—offer an excellent model to test the capacity of embryonic PCs to interact with the neural elements of the impaired adult circuit of the mutant cerebellum, to replace the missing PCs of the host. The model system we chose was the replacement of missing PCs in the cerebellum of the *Purkinje cell degeneration* (*pcd*) mutant strain of mice (Mullen et al. 1976). *Nna1,* a gene encoding a putative

nuclear protein containing a zinc carboxypeptidase domain, is the mutated gene in the *pcd* mouse (Fernandez-Gonzalez et al. 2002). In this mutant, the PC degeneration takes place after weaning, when most of the developmental process of the cerebellar cortex and the corticonuclear connection are finished (Sotelo 2004). At 2 months, less than 1 % of the normal PC population remains, and by 4 months almost all have degenerated, remaining an average of 107 PCs in the whole cerebellum, mainly located in the nodulus (Wassef et al. 1986).

Sotelo and Alvarado-Mallart (1986) first described how grafted PCs migrate into the molecular layer of the host cerebellum replacing the missing neurons of the *pcd* mutant. Following the time course of the development of the embryonic day 12 (E12) cerebellar primordium into the postnatal day 60 (P60) cerebellum, it was possible to show the stages followed by the grafted PCs to be integrated in the cortical circuitry of the adult host cerebellum 4 days after transplantation (Sotelo and Alvarado-Mallart 1987). It was evident that the grafted PCs begin migrating to their proper domain into the host cerebellum, and even though they do not become aligned in a row as in the control cerebellum, they reach their proper environment dispersed within the host molecular layer. This migration reaches its maximal rate 2–3 days later, and the arrest of the migration takes place in 7 days, indicating that the attractive influences which manage the migration are present even in the adult cerebellum, but the migratory potential of the immature cells weakens in a precise developmental stage either during normal development or after grafting, as if provided with an internal clock. Once in the molecular layer, grafted PCs develop dendritic trees appropriately oriented, perpendicular to the parallel fibers of the host cerebellar cortex, and receive with precision the different inputs not only from cerebellar interneurons (parallel fibers, basket cell axons, and stellate cell axons) but also from extracerebellar climbing fibers with which they establish normal synaptic connections at the appropriated location of the dendrites.

The above-summarized results clearly show that neurons and axons of the host brain retain their capacity for synaptogenesis into adulthood and, similarly, that embryonic grafted neurons are able to pursue their inner tempo for migration and differentiation. In addition, the embryonic grafted PCs by interacting with the adult host neural cells (namely, Bergmann fibers, cerebellar interneurons, relay granule cells, and inferior olivary neurons) have the capacity to become synaptic partners for adult axon terminals. The newly formed synapses are functional as early as 10 days after grafting, when excitatory as well as inhibitory responses were already elicited in these cells by electrical stimulation of the host subcortical white matter (Gardette et al. 1990). Therefore, while the synaptogenesis with excitatory axons occurs in synchrony with that happening during normal development, the one with inhibitory axons occurs much faster. Despite this accelerated synaptogenesis PCs, like in normal development, pass through a phase of multiple innervation by climbing fibers (Crepel et al. 1976), transient phase that takes place also with an advanced time course, since by 15 days after grafting (equivalent to P8 aged PCs) it is already finished. These results clearly emphasize that the embryonic and adult neurons confronted in the transplantation experiments are provided with an extraordinary plastic capacity allowing the proper integration of grafted PCs in the deficient circuit of

the *pcd* cerebellar cortex. With this great potentiality for plasticity, the obvious question is to determine if the cerebellar grafts can improve the motor ataxia in the *pcd* mice. The answer is that one essential prerequisite remained to be fulfilled before a functional and behavioral improvement could be expected. Indeed, although the deficient circuitry of the cerebellar cortex has been repaired with the synaptic integration of the grafted PCs, the cortico/nuclear projection remains constantly deficient, and only in a few occasions we have been able to disclose a few PC terminals synapsing upon deep cerebellar nuclear neurons.

Nevertheless, the obtained results concerning the integration of the grafted PCs in the cortical circuit are very interesting for our purposes of morphological neural plasticity. Indeed, they reveal a new kind of neural plasticity, the one required for the possible interactions among neural cells of similar phenotypes but of complete different ages, such as young postmitotic with adult neurons. The timing and the nature of the cellular interactions involved in the migration of grafted PCs into the host cerebellum should be similar to those that take place during normal ontogeny, suggesting that, in both cases, the developmental program is regulated autonomously by the participating cells, independent of external factors, as regulated by an internal clock (Sotelo and Alvarado-Mallart 1987; Sotelo et al. 1994). Although little is known about the molecular identity of the signals involved, it is clear that they are developmentally regulated and transiently expressed during ontogenesis, but what about mutually interacting molecular signals when embryonic neurons are grafted into adult brain? This raises the question as to whether the grafted embryonic PCs induce adult host cells, in particular the Bergmann glia, to transiently reexpress the molecular signals needed for their migration and synaptic integration into the host (Sotelo and Alvarado-Mallart 1987) or whether isochronic embryonic astrocytes, bearing the appropriate factors because of their age, also migrate from the graft and acquire the Bergmann fiber phenotype, providing this way substrate for migration of immature PCs. Transplant experiments carried out with donor embryonic cerebellum taken from a transgenic mouse line, which shows an ectopic expression of the hybrid Krox-20/lacZ gene in Bergmann fibers, allowed us to determine that the Bergmann fibers subserving radial migratory pathways to grafted PCs do not originate from the graft and, therefore, that they belong to the host (Sotelo et al. 1994). Moreover, in the developing cerebella the embryonic Bergmann fibers, the migratory substratum to immature PCs, like all other radial glia, express nestin an intermediate filament (Lendahl et al. 1990). This expression is transient, and once all the migratory processes are completed in the molecular layer (PCs, granule cells, and inhibitory interneurons), the expression of nestin is lost. Using a monoclonal antibody against nestin, we were able to reveal that only those Bergmann fibers within the cortical areas containing either the grafts or, particularly, grafted PCs migrating into the molecular layer reexpressed nestin.

The observed spatiotemporal correlation between grafted PC radial migration and nestin expression in the host molecular layer validates that these grafted embryonic neurons induce nestin expression in adult glial fibers. The molecular identities of the signals involved in such kind of interaction remain to be determined. Therefore, despite the actual absence of molecular mechanisms, it was possible to

establish that grafted embryonic neural cells can interact with adult neural cells through a new and interesting plastic mechanism that regulates gene expression in neural cells of the adult host recapitulating transient phenotypes associated with specific stages of normal development. This new type of morphological plasticity was named "adaptive rejuvenation." The occurring changes transiently generate a microenvironment permitting radial migration of the PCs, a necessary prerequirement for their synaptic integration and the restoration of normal circuitry in the cerebellar cortex of pcd mice.

Transplantation of cerebral cortical embryonic cells into lesioned rodent cortex, initiated by Michel Roger and Afsaneh Gaillard (1998), has provided interesting results. Indeed, as published by Gaillart et al. (2007), when embryonic neocortex taken from transgenic mice overexpressing a green fluorescent protein was grafted into damaged murine adult motor cortex, grafted neurons were able to send axonal projections to specific target nuclei in the motor thalamus and even very long projections to the spinal cord. These important and unexpected results suggest that neural cell transplantation might promote the reconstruction of damaged circuits after adult brain injury. In addition, Pierre Vanderhaegen, Afsaneh Gaillard and collaborators have more recently shown that embryonic stem cells and induced pluripotent stem cells (iPSCs), cultured without added morphogens, can by themselves replace cortical neurons (Gaspard et al. 2008; Espuni-Camacho et al. 2013). These experiments open a new aspect of the requirements of transplanted precursor cells for the integration and repair of adult brain circuits.

2.6 Concluding Remarks

In conclusion, the data reported in this review, although only represent a low percentage of the very numerous interesting papers published on this topic, are aimed to emphasize how the important concept of structural neuronal plasticity has accompanied neuroscientists from the middle of the nineteenth century, even before the appearance of the "neuron doctrine," considered as the milestone marking the beginning of modern neuroscience. The concept built up by the pioneer workers cited in this review implies that the nervous system is in a continuous remodeling process, by destroying and building up neuronal connections that allow us to consider the brain as a "neotenic" organ. Despite the enormous interest of the asked questions, and the major breakthroughs obtained in recent years as a result of the refinement of cellular imaging and transgenic methods, many of these old questions reviewed here still remain unanswered. For example, what is the real meaning of the maintenance of postsynaptic differentiations occurring only in a portion of the deafferented synapses? Are they indicative of the presence of neurotransmitter receptors or only of their scaffolding proteins? What are the possible deleterious effects of heterologous reafferentation by terminal axonal sprouting? How to get the matching of the new neurotransmitter, frequently GABA, and the former receptor that in most of the examples of eliminated synapses was a glutamate receptor? Hopefully with

the arrival of new technologies, new vocations for the structural study of the nervous system will continue to emerge among young researchers, and these young researchers become interested in pursuing the evolution of these concepts and the resolution of old problems.

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Chapter 3
Impact of Injured Tissue on Stem Cell Fate

Bobbi Fleiss, Vibol Chhor, Luigi Titomanlio, Stéphane Auvin, Olivier Baud, and Pierre Gressens

Abbreviations

DAMPs	Damage-associated molecular patterns
EPO	Erythropoietin
HI	Hypoxia–ischaemia
OPC	Oligodendrocyte progenitor cell
RMS	Rostral migratory stream
SGZ	Sub-granular zone
SVZ	Subventricular zone
TBI	Traumatic brain injury
TLR	Toll-like receptor
VZ	Ventricular zone

3.1 General Concept of Post-lesion Plasticity

Acute brain insults such as hypoxia–ischaemia (HI) or traumatic brain injury (TBI) are associated with high rates of morbidity and mortality, despite the massive search for neuroprotective strategies over the past few decades. One reason for this is the fact that once the cellular and molecular cascade of events leading to brain damage

B. Fleiss • V. Chhor • P. Gressens (✉)
French Institute of Health and Medical Research, U1141, Paris Diderot University, Sorbonne Paris Cité, PremUP, Paris, France
e-mail: pierre.gressens@inserm.fr

L. Titomanlio • S. Auvin • O. Baud
French Institute of Health and Medical Research, U676, Paris Diderot University, Sorbonne Paris Cité, PremUP, Paris, France

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3_3,

is fully initiated, it becomes very difficult if not impossible to block it. This is well illustrated by the demonstration that, in term neonates with HI encephalopathy, hypothermia has to be initiated within the first 6 h after birth to have a significant neuroprotective effect (Edwards et al. 2010). Similarly, thrombolysis with tissue-type plasminogen activator, the only approved treatment for acute ischaemic stroke in adults, has to be instituted within the first 4.5 h of stroke (Carpenter et al. 2011). In this context, fostering positive post-lesion plasticity appears to be a very promising strategy for delayed interventions aimed at improving long-term neurological and cognitive function (Fig. 3.1).

Although our knowledge of the cellular and molecular mechanisms underlying post-lesion brain plasticity is still limited, the injury-induced proliferation of neural stem/progenitor cells appears to be an important attempt at self-repair by the damaged brain. Understanding the mechanisms underlying this post-insult proliferation of neural stem/progenitor cells, their survival, migration and differentiation into different types of neural cells, and ultimately, their integration into functional networks would allow for tailor-made intervention strategies to be designed to convert this endogenous response of the damaged brain into a more efficient repair mechanism and leading to the long-term improvement of brain function (Fig. 3.2).

While focusing mainly on acute diseases affecting the developing brain, the present chapter will review recent knowledge on the impact of brain damage on neural stem/progenitor cell fate, on the underlying mechanisms, and on promising experimental strategies aimed at improving the effectiveness of this self-repair mechanism. From a translational point of view, it is important to note that, to our knowledge, there are as yet no solid data concerning the impact of injury on stem cell fate in the developing human brain, a question that could be addressed by studying post-mortem brain tissue. Similarly, studies in non-human primates with acute brain damage would yield invaluable information.

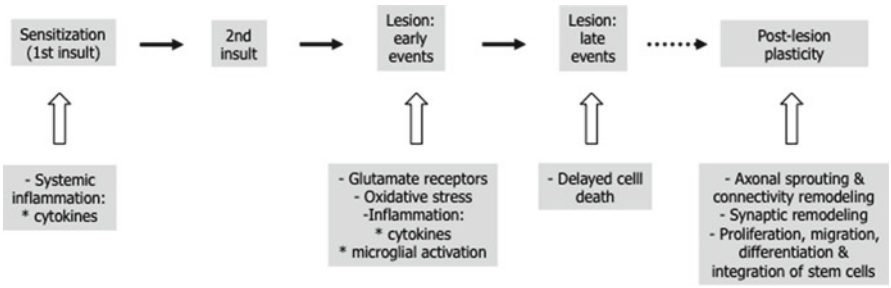

Fig. 3.1 Schematic representation of the potential targets for neuroprotection of the developing brain

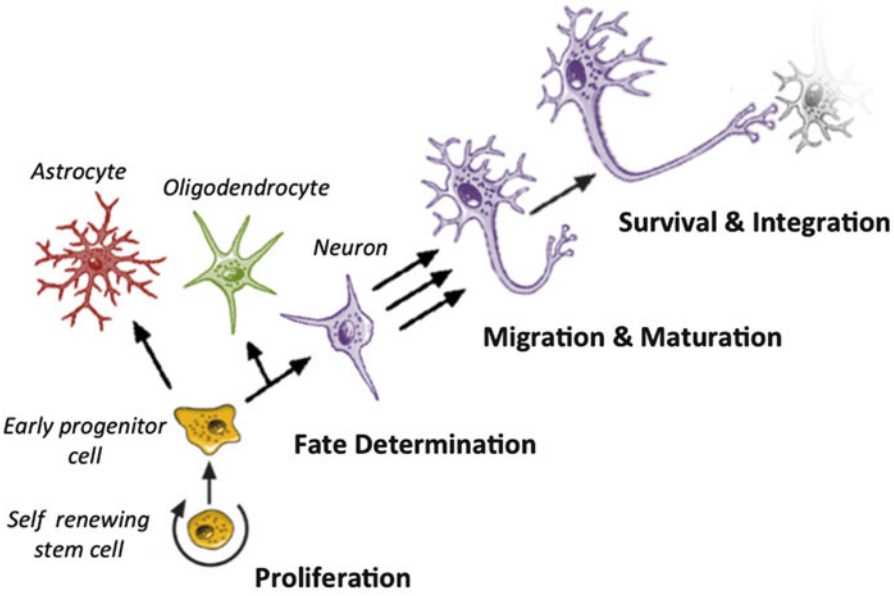

Fig. 3.2 Schematic representation of the four stages between proliferation and successful integration of endogenous stem cells following brain insult

3.2 Modelling Acute Damage to the Developing Human Brain in Animals

In an attempt to model diseases of the developing brain, researchers have developed two complementary approaches: (1) some models use animals to test, in a well-defined experimental setting, the causal link between the potential triggering event and the outcome; (2) other models attempt to decipher the underlying cellular and molecular mechanisms in a reductionist setting, potentially allowing the identification of new biomarkers and/or targets for neuroprotection. The latter studies do not directly address the causal link and often rely on toxins or pharmacological agents.

Although brain damage in preterm and term neonates, juvenile traumatic brain injury, and developmental epilepsy are very distinct entities, they share some common cellular and molecular mechanisms such as excitotoxicity and neuroinflammation (involving, among others, microglial activation, pro-inflammatory cytokines and chemokines, and toll-like receptor—TLR activation through the production by injured neural tissues of damage-associated molecular patterns—DAMPs/alarmins). This is the generally accepted rationale for using excitotoxic agents (such as agonists of NMDA or AMPA–kainate glutamate receptors), pro-inflammatory cytokines, or

TLR agonists to mimic certain mechanistic aspects of these human brain disorders in animals (Czeh et al. 2011; Degos et al. 2010; Hagberg et al. 2002; Kaindl et al. 2009).

Term newborns and very premature infants (<32 weeks of pregnancy) are at risk for the development of brain damage, which can be detected with brain imaging and which is responsible for motor, cognitive, and behavioural sequelae (Himmelmann et al. 2005; Marlow et al. 2005; Marret et al. 2013). The lesions found in the term neonate generally affect the grey matter (cortex and grey nuclei), while the periventricular white matter is the principal site of cerebral lesions detected in the premature infant. Recent imaging studies have shown that certain premature infants also exhibit cortical grey matter injury, the histological characteristics of which remain to be fully elucidated (Ball et al. 2013). In the field of perinatal brain damage, while there is no definitive consensus as to the cause of brain damage, two major hypotheses predominate: that of an HI origin and that of an inflammatory origin. Accordingly, various animal models have been developed based on acute HI insults (transient umbilical cord occlusion during late gestation or carotid artery ligation combined with transient hypoxia in the early neonatal period), chronic hypoxic insults (protracted isolated hypoxia during either the fetal or early postnatal period), or acute/subacute inflammatory insults (LPS, PolyI:C, or IL-1-beta administration during either the late fetal or early postnatal periods) (Hagberg et al. 2002; Ramanantsoa et al. 2013).

Human epilepsy is defined by the appearance of multiple spontaneous recurrent seizures. As such, induction of acute seizure activity alone without subsequent chronic seizures should be regarded as a model for seizures per se, and not epilepsy (Auvin et al. 2012). When studying epilepsy syndromes, the use of immature animal allows us to study facets of epileptogenesis, the progressive biochemical, anatomic, and physiologic changes that lead to spontaneous recurrent seizures. Spontaneous seizures develop after a "silent" period that may last between several days and several months, and this latent period is a major challenge for modelling paediatric epileptogenesis as laboratory rodents progress from the neonatal to the adult age in few weeks instead of many years, as happens in humans. Therefore, by the time spontaneous seizures occur and observations of the effects on brain development can be made, the animal has already passed through several developmental stages, and often the animals display spontaneous recurrent seizures during the adulthood.

Whether cell injury is required in the immature brain to induce epileptogenesis remains an active topic of debate, and different types of injury and/or prolonged seizure have been used to induce epileptogenesis in order to mimic facets of the human conditions. Hyperthermic seizure induced in P10–P11 rat pups is used to mimic prolonged febrile seizure (Dube et al. 2006). Hypoxic–ischemic brain insults can be also induced in rat pups (at postnatal day 7–10) in order to model term hypoxic–ischemic encephalopathy (Kadam et al. 2010; Rakhade et al. 2011) that is a prominent cause of mortality in neonate and morbidity including epilepsy in children (Wallace 2001). Status epilepticus induced by pilocarpine is used from postnatal day 14 to adult rat to induce a significant epileptogenesis and result in spontaneous seizures (Raol et al. 2003; Sankar et al. 1998).

TBI is difficult to model as, in humans, it can result from various mechanisms and thus has a complex pathophysiology (Morganti-Kossmann et al. 2010). Focal

brain damage can lead to either contusion or intracranial haemorrhage, whereas diffuse brain damage induces diffuse axonal injury and brain swelling. Actually, most of the time in humans, both lesions are observed. Various animal models have been developed to induce focal lesions, by an air-driven piston (controlled cortical impact) or water (lateral fluid percussion) directly onto the dura, by dropping a weight such as described in the Feeney weight-drop model or by applying a cold rod (cryogenic injury model) to the exposed dura or directly to the skull. On the other hand, diffuse lesions are assessed by modifying the protocols described above to include a central point of impact, such as by central fluid percussion or the dropping of a weight onto a helmet or an injury plate placed on the central area of the skull in the Marmarou weight-drop model (Prins and Hovda 2003).

3.3 Effects of Injury on Stem Cells

3.3.1 Impact of Hypoxia–Ischaemia

Several groups have studied the impact of permanent unilateral carotid artery ligation combined with transient hypoxia (HI) on endogenous stem cell proliferation and fate in newborn rodents. The range of postnatal ages used in these studies roughly corresponds to 34–42 GW in humans, which thus mostly mimic lesions observed in term infants with HI encephalopathy. Accordingly, the pattern of lesions observed in these studies mainly involves the neocortex, hippocampus, and deep nuclei.

These studies have consistently shown an increase in the number of neural stem/progenitor cells in the medial subventricular zone (medial SVZ, which contains neural stem/progenitor cells that are resilient to HI) following HI (Felling et al. 2006; Kadam et al. 2008; Romanko et al. 2007; Yang et al. 2007; Yang and Levison 2006, 2007), while numerous neural progenitors die in the lateral SVZ in the same animals (Romanko et al. 2007). Neurospheres derived from the HI-exposed medial SVZ generate three times as many neurons and twice as many oligodendrocytes in vitro when compared to control neurospheres (Yang and Levison 2006).

Although it is widely accepted that this increased proliferation in the SVZ leads to the migration of newly formed neural cells towards the striatum (Felling et al. 2006; Kadam et al. 2008; Yang and Levison 2006), the migration of SVZ-derived neural cells to the neocortex is more controversial (Donega et al. 2013). While several groups have failed to demonstrate the significant migration of SVZ-derived cells after HI, the Levison group has unambiguously demonstrated that neonatal HI in 6-day-old Wistar rats induces the migration of neurons from the SVZ towards the injured neocortex (Yang et al. 2007). One potential mechanism for this increased migration is the diversion of some SVZ-derived cells that normally migrate towards the olfactory bulbs through the rostral migratory stream (RMS) to the injured neocortex following HI. Interestingly, it has recently been shown that, in the normal developing human brain, some migrating cells from the RMS are diverted and

migrate towards the frontal neocortex (Sanai et al. 2011). Using an organotypic slice culture model, the Kiss group has also shown that GFP-labelled SVZ-derived progenitor/stem cells transplanted into the SVZ of newborn rats migrate into the neocortex and differentiate into GABAergic neurons (Dayer et al. 2008). The discrepancies between these various reports might be related to differences in the HI paradigm (duration and depth of hypoxia leading to graded severity of brain damage) or the species (mice vs. rats) or rat strain used (Sprague–Dawley vs. Wistar rats). Further studies will be necessary to address this key point before the application of these results to regenerative medicine in the perinatal field.

In studies of the impact of perinatal HI on hippocampal neurogenesis, a reduction in the proliferation in sub-granular zone (SGZ) cells of the dentate gyrus has generally been reported (Kadam et al. 2008). SGZ cells are generally considered lineage-restricted progenitors rather than neural stem/progenitor cells, which might explain why they are not as resilient to HI as neural progenitors in the lateral SVZ (Romanko et al. 2007; Covey et al. 2011).

Post-HI inflammatory mediators and growth factors seem to play an important role in the HI-induced proliferation of SVZ neural stem/progenitor cells. IL-6 and LIF are induced in the SVZ by neonatal HI and enhance neurosphere growth, self-renewal, and tripotentiality (Covey et al. 2011; Buono et al. 2012; Covey and Levison 2007). The effects of LIF are mediated, at least in part, by the Notch pathway (Felling et al. 2006; Covey and Levison 2007). It has also been shown that HI induces the increased expression of EGFR in the SVZ, leading to increased sensitivity to EGF and a subsequent increase in the proliferation of SVZ neural stem/progenitor cells (Alagappan et al. 2009). In a very elegant study, the Götz group has recently shown that the molecular signal sonic hedgehog is necessary and sufficient for invasive injury (such as ischaemia or stab wounds) to elicit the proliferation of astrocytes in the adult cerebral cortex that can form self-renewing and multipotent spheres in vitro (Sirko et al. 2013). Further studies are necessary to determine if this is also the case in the developing brain.

Despite the increased production of neural stem/progenitor cells in the neonatal HI-exposed SVZ, several factors limit the impact of this increased neurogenesis on outcome. (1) The differentiation of these cells is biased towards the astroglial lineage, with relatively few new neurons and oligodendrocytes produced. The microenvironment of the SVZ following HI is evidently different from that of the SVZ during normal brain development. Recent studies have started to identify some of the local factors produced by the SVZ that are important for the commitment of newly generated progenitors following neonatal HI. VEGF-A and TGF-beta1 (through ALK5 signalling) in the SVZ promote the production of cells committed to the astroglial lineage while VEGF-C promotes the production of cells committed to the oligodendroglial lineage (Bain et al. 2010, 2013). (2) The migration of newly generated cells towards damage sites is not optimal. Studies with stem cells transplanted following brain damage suggest that chemokines such as SDF-1 and its receptors might also play a key role in the migration of neural cells derived from the SVZ following HI. In addition, trophic factors such as VEGF, acting on progenitor

cells through the expression of VEGFR2, play a role in the migration of SVZ-derived cells (Zhang et al. 2003). Optimising the molecular cues controlling migration might help improve the effectiveness with which cells generated in the SVZ are directed towards the damage sites where they are required. (3) Most studies have shown the limited survival of neurons and oligodendrocytes produced in the post-HI SVZ. The local environment in injured brain areas is very different from that of normally developing brain structures and most likely does not provide the molecular cues necessary to support the survival of newly produced neurons or oligodendrocytes. Attempts have recently been made to further characterise this unfavourable milieu and to provide better conditions to promote the survival of SVZ-derived cells following HI (see below). (4) Similarly, studies have shown that the integration of newly generated neurons into existing networks is limited, restricting their functional benefits. Evidently, there is a direct link between this limited integration and the poor survival of newly produced cells, as neurons not integrated into networks would have a decreased chance of survival.

3.3.2 Impact of Chronic Hypoxia

Chronic hypoxia during late gestation or early postnatal life has been used in rodents to produce diffuse cortical neuronal loss and/or periventricular white matter damage, potentially mimicking the brain injury observed in preterm infants (Hagberg et al. 2002).

Exposing newborn mice to hypoxia between P3 and P11, the Gallo group has demonstrated that, following hypoxia-induced oligodendrocyte cell death in the periventricular white matter, there is a phase of oligodendrocyte progenitor cell (OPC) proliferation both in the SVZ and in the white matter itself (Jablonska et al. 2012). The proliferation of progenitors in the SVZ lasted longer than in the white matter, suggesting distinct mechanisms. Indeed, the authors have shown the involvement of the Cdk2-FoxO1-p27^{Kip1} pathway in the white matter and the Cdk4 pathway in the SVZ. Despite this increased proliferation of OPCs, the newly produced oligodendrocytes failed to produce normal myelin (as demonstrated by the lower *g*-ratio of myelinated axons), indicating that hypoxia induces delayed OPC maturation. This failure to produce normal myelin despite the higher number of oligodendrocytes suggests that timing is crucial for optimal myelination, as crosstalk between developing axons and maturing oligodendrocytes is central to the process of myelination.

Using a similar model, the Vaccarino group has shown that hypoxia induces a 30 % decrease in cortical neurons (Fagel et al. 2009). Hypoxia-exposed animals displayed a twofold increase in cell proliferation in the SVZ and a threefold increase in proliferation in the olfactory bulbs. This proliferative response was blunted in mice with a deletion of Fgfr1 in GFAP-positive cells (conditional KO mice). One month after the hypoxic insult, the number of excitatory cortical neurons was restored while there was still a partial deficit in cortical GABAergic interneurons.

3.3.3 Impact of Neuroinflammation

The effects of post-HI neuroinflammation on neural stem cells have been described above. In contrast, little information is available on the effects of systemic inflammation, neuroinflammation being used to model the chorioamnionitis frequently observed in preterm neonates and considered a major risk factor for an unfavourable neurological outcome (Dammann and Leviton 1997). However, one recent study has elegantly shown that the exposure of pregnant mice to one injection of LPS on E13.5 (approximately equivalent to 25 weeks GA) induces a significant reduction in cell proliferation in the VZ of the developing cortex (Stolp et al. 2011). This reduced proliferation is accompanied by a change in the mode of division (change of angle of the mitotic spindle and of beta-catenin distribution) suggestive of a switch from symmetric to asymmetric division. When studied at P8, the cortex of LPS-exposed animals showed an abnormal neuronal density in layers IV and V. Long-term histological and behavioural studies will be necessary to determine the consequences of this reduced proliferation in the VZ. In addition, it will be interesting to determine the impact of a similar inflammatory insult on the SVZ at later stages of brain development.

3.3.4 Impact of TBI

Following the Kennard principle, injuries affecting the developing brain generally have a better prognosis than similar injuries occurring in the adult brain (Kennard 1936). However, TBI seems to be an exception to this general principle, with children exposed to TBI displaying a poorer long-term outcome, suggesting that TBI significantly impairs endogenous mechanisms of plasticity in the developing brain (Bonnier et al. 1995). The mechanisms involved, however, remain unspecified. One potential hypothesis is that TBI has a negative effect on endogenous stem cells in the developing brain. Unfortunately, to our knowledge, few studies have directly addressed this intriguing question in the developing brain though numerous studies have provided significant insight into stem cell remodelling following TBI in the more mature adolescent brain (see Chap. 4).

In a cryogenic model of somatosensory cortex damage mimicking TBI in the developing brain, it has been shown that TBI induces increased proliferation in the SVZ, but this effect is mostly observed when the TBI occurs at P6 and is much less pronounced when it occurs at P14 (Covey et al. 2010). In addition, as compared to HI, in which proliferation in the SVZ remains higher at least up to 2 months post-insult, the post-TBI increase in proliferation rapidly fades away. If transposable to humans, this study would suggest that post-TBI proliferation in the SVZ is restricted to early stages of brain development and that this increased proliferation, when it occurs, is limited temporally following the insult. Notably, the capacity for post-TBI proliferation in the SGZ peaks in the juvenile (P28) rat brain (compared with

P90) and up to 70 % of new cells go on to express a marker for mature neurons (Sun et al. 2005), suggesting that there are important differences in the cues available to these populations to support endogenous repair. An in vitro model of TBI using developing brain sections has identified sonic hedgehog as a key molecular player underlying TBI-induced proliferation in the SVZ (Ahmed et al. 2012). Further studies of these developmental processes and differences are critical to developing cell-based therapies for the paediatric population at risk of TBI.

3.3.5 Impact of Epilepsy

In the developing brain, studies looking at the impact of epilepsy on neurogenesis have focussed on the dentate gyrus in rodents. As recently reviewed by Porter (2008), the effect of seizure on dentate gyrus neurogenesis is complex and largely dependent upon the age at which the seizures are, the seizure model used induced (seizure or status epilepticus, model), and the time at which neurogenesis is evaluated (ictal, postictal, or at delayed time points). Although it is difficult at this stage to draw firm conclusions, Porter has elegantly summarised the published data as follows (Porter 2008) (for further details, please refer to Table 1 in Porter (2008)): (1) seizures elicited during the first postnatal week in rodents induce decreased postictal neurogenesis in the dentate gyrus without affecting the maturation of newly formed cells; (2) seizures elicited during the second postnatal week, depending on the model, induce no change or a mild increase in neurogenesis; in addition, in some models, they increase the survival of newly formed neurons; (3) seizures elicited during the third and fourth postnatal weeks increase neurogenesis, following the pattern observed in adult animals. Clearly, more studies are needed to further understand these complex effects of seizure on neurogenesis in the dentate gyrus of the developing brain and their functional consequences (epileptogenesis and/or cognitive dysfunctions). Similarly, it would be interesting to investigate in detail the effects of seizures on neurogenesis in the SVZ. This topic is addressed in more detail in Chap. 5.

3.4 Role of Exogenous Trophic Factors and Exogenous Stem Cells

As discussed above, despite the increased production of neural stem/progenitor cells in the developing SVZ exposed to invasive insults such as HI, the limited survival, differentiation, and integration of these newly produced cells limit their impact on outcome. To address this issue, two main strategies have been tested experimentally: the use of trophic factors and exogenous stem cells.

In proof-of-concept studies, the overexpression of FGF-2 in neural progenitor cells enhanced their proliferative and migratory characteristics, including following

HI (Dayer et al. 2007; Jenny et al. 2009). Similarly, erythropoietin (EPO) administered after neonatal HI increased the percentage of newly produced neurons while decreasing newly produced astrocytes in the SVZ (Gonzalez et al. 2007). This effect of EPO was accompanied by a significant long-term preservation of hemispheric volume. In an in vitro model of HI insult (oxygen–glucose deprivation), EPO displayed similar effects on hippocampal neurogenesis (Osredkar et al. 2010). In addition, an EPO derivative administered 5 days after neonatal HI promoted the maturation of SVZ-derived OPCs and enhanced neurological function (Kako et al. 2012). Following neonatal HI, osteopontin deficiency (KO mice) decreased the HI-induced proliferation/survival of OPCs without affecting the neuronal lineage (van Velthoven et al. 2011), suggesting that osteopontin could be another strategy to target SVZ-derived OPCs and improve outcome.

Notably, rehabilitation has been shown to have positive but incomplete effects on neurological outcome following damage to the developing brain. For example, children with cerebral palsy receiving constraint-induced movement therapy for 21 days were shown to acquire more new classes of motor skills (9.3 vs. 2.2 in control children with cerebral palsy) and to have an increased use of the more-affected arm in a laboratory motor function test (52.1 % vs. 2.1 % of items) (Taub et al. 2004). Such a rehabilitation therapy was associated with a significant increase in grey matter volume in the sensorimotor cortex contralateral to the more-affected arm (Sterling et al. 2013). Despite these recent studies, the mechanisms by which rehabilitation works are globally poorly understood, although such knowledge could permit better-targeted interventions and further improve outcome. Based on the fact that environmental enrichment in rodents has been shown to increase trophic factor production and neurogenesis in the hippocampus (Bekinschtein et al. 2011), one could hypothesise that rehabilitation increases the production of some trophic factors in the SVZ and SGZ and thus favours endogenous neurogenesis, leading to improved function.

In most experimental models, the administration of exogenous stem cells after brain damage has generally been a failure in terms of the survival, differentiation, and integration of the grafted cells (Titomanlio et al. 2011). However, several studies have shown a benefit of this strategy on long-term outcome (Titomanlio et al. 2011). Different mechanisms have been proposed for this limited success, including (1) anti-inflammatory effects (especially for mesenchymal stem cells), (2) the secretion of factors that make the environment more favourable for endogenous cells, and (3) the transfer of healthy mitochondria to damaged endogenous brain cells. These mechanisms could have a potential impact not only on already differentiated neurons and oligodendrocytes that are struggling to survive and adapt to brain damage but also on newly generated cells in the SVZ or SGZ. This latter point has been recently demonstrated by the Heijnen group, who significantly increased the production of neurons and oligodendrocytes in the cortex and hippocampus by administering mesenchymal stem cells 3 days or 3 and 10 days after neonatal HI (van Velthoven et al. 2010a, b). Further discussion on this exciting topic can be found in the excellent recent review from the Heijnen group (Donega et al. 2013).

3.5 Conclusion

In conclusion, we have an incomplete but steadily increasing understanding of the processes required for endogenous stem cell proliferation to result in improvements in brain function after injury. Most often this process includes differentiation, migration, and integration—with neuronal specification appearing to most benefit the brain. It has been a fairly recent realisation that the responses of the developing and adult brain to injury are so disparate and it is from this new understanding that we are beginning to compare and contrast with the great wealth of information from adult studies. Harnessing natural repair functions must be of primary interest for all fields of brain injury research as endogenous stem cells provide us with benefits from exogenous therapies that are numerous and include autologous and ethically unchallenging cell origins and hopefully a lower technical burden, increasing accessibility to new therapies for the many babies and children suffering brain injury in low resource settings.

Acknowledgements The research of the authors is supported by the INSERM, the Paris Diderot University, the PremUP Foundation, the Seventh Framework Programme of the European Union (grant agreement No. HEALTH-F2-2009-241778/Neurobid), the Indo-French Centre for the Promotion of Advanced Research (CEFIPRA project No. 4903-H), the Leducq Foundation, the de Spoelberch Foundation, the Grace de Monaco Foundation, and the Assistance Publique-Hôpitaux de Paris ("contrat hospitalier de recherche translationnelle" to Dr. Pierre Gressens).

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Chapter 4
Role of Neural Stem and Progenitor Cells in the Adaptation of the Brain to Injury

Sue Hong, Tzong-Shiue Yu, and Steven G. Kernie

Abbreviations

AED	Antiepileptic drug
Ara-C	Cytosine-B-D-arabinofuranoside
BrdU	Bromodeoxyuridine
CBF	Cerebral blood flow
CCI	Controlled cortical impact
DCX	Doublecortin
Epo	Erythropoietin
EpoR	Erythropoietin receptor
FGF	Fibroblast growth factor
FPI	Fluid percussion injury
GFAP	Glial fibrillary acidic protein
HIE	Hypoxic–ischemic encephalopathy
HSV-TK	Herpes simplex virus thymidine kinase
MCAO	Middle cerebral artery occlusion
MCM2	Minichromosome maintenance protein
MWM	Morris water maze
NSPC	Neural stem/progenitor cell
NSE	Neuron-specific enolase
PCNA	Proliferating cell nuclear antigen

S. Hong • T.-S. Yu • S.G. Kernie, M.D. (✉)
Departments of Pediatrics and Pathology & Cell Biology,
Columbia University College of Physicians and Surgeons,
630 W. 168th Street, P&S Building, Rm 11-432, New York, NY 10032, USA
e-mail: sk3516@columbia.edu

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3_4, © Springer Science+Business Media New York 2014

PSA-NCAM	Polysialylated neural cell adhesion molecule
SGZ	Subgranular zone
SVZ	Subventricular zone
TBI	Traumatic brain injury
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor

4.1 Adult Neurogenesis

In the human brain, neural stem and progenitor cells (NSPCs) from the subventricular zone (SVZ) of the lateral ventricles and subgranular zone (SGZ) of the hippocampal dentate gyrus give rise to new neurons throughout life. While this persistent ability to make new neurons has been most extensively studied, these multipotent neural stem/progenitor cells also give rise to multiple cell types, including astrocytes and oligodendrocytes (Zhao et al. 2008). Since oligodendrocyte and glial progenitors are being discussed in detail in Chap. 7 by J. M. Mangin and Chap. 8 by P. Ballabh, the focus here will be on injury-induced neurogenesis from the SGZ and SVZ (Fig. 4.1).

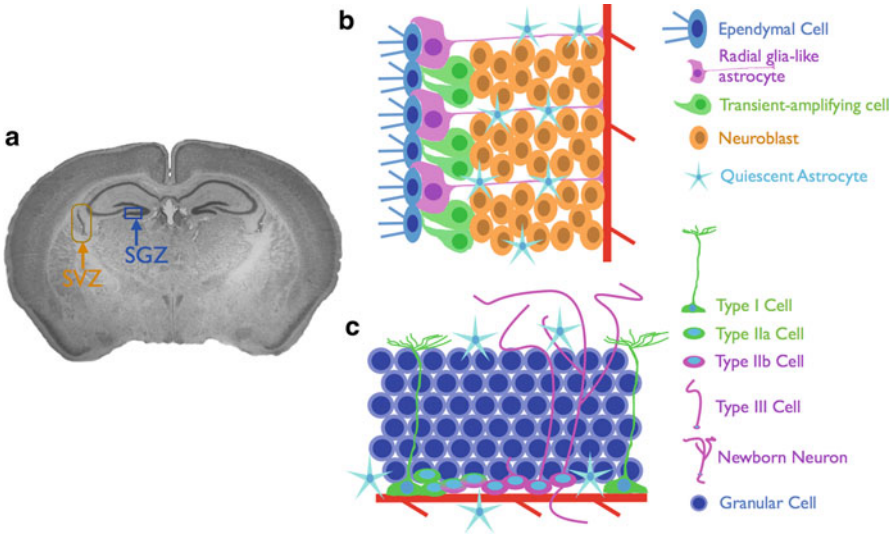

Fig. 4.1 Coronal section of mouse brain depicting the neurogenic subventricular zone (SVZ) and subgranular zone (SGZ) of the hippocampus (**a**). The SVZ consists of a variety of closely opposed cell types including the ependymal cells lining the cerebral ventricle, radial glial-like astrocytes representing stem cells, the actively proliferating transit amplifying cells, committed neuroblasts, and quiescent astrocytes (**b**). The SGZ consists of type 1 stem cells, type 2a rapidly proliferating neuroblasts, type 2b migrating neuroblasts, type 3 committed process-extending neuroblasts, new neurons, granular neurons, and quiescent astrocytes (**c**)

4.1.1 Neurogenesis in the Subventricular Zone of the Lateral Ventricles

Within the subventricular zone, neural stem cells express specific markers indicative of their ability to both self-renew and become mature neural lineage cells such as astrocytes and neurons. Stem cells in the SVZ express both nestin and glial fibrillary acidic protein (GFAP) and upon asymmetric division give rise to doublecortin-expressing neuroblasts that migrate in chains through the rostral migratory stream to the olfactory bulb, where they differentiate into granule cells and perigranular interneurons (Doetsch and Alvarez-Buylla 1996). In the olfactory bulb, many of these newly generated progenitor cells and young neurons undergo apoptosis, while others differentiate into neurons and incorporate long term into the olfactory bulb (Petreanu and Alvarez-Buylla 2002). The long-term survival of these newly generated neurons is influenced by sensory input, which in rodents is likely responsible for relatively quick adaptations to different olfactory stimuli (Petreanu and Alvarez-Buylla 2002). Neurogenesis via the SVZ–olfactory bulb system continuously replaces preexisting cells and increases cell numbers over time, while sustaining a continuous supply of SVZ-resident stem/progenitors that are a potential reservoir for other non-olfactory functions (Kernie and Parent 2010). In early postnatal life, these neural stem/progenitors cells may migrate beyond the olfactory bulb to contribute neurons in the cortex and subcortex, possibly migrating along blood vasculature (Inta et al. 2008; Magueresse et al. 2012). The role of this persistent neurogenesis in the olfactory bulb is currently not fully known, but may contribute to olfactory memory and discrimination. This phenomenon is likely dependent upon age as well as duration of loss of neurogenesis (Imayoshi et al. 2008).

4.1.2 Neurogenesis in the Subgranular Zone (SGZ) of the Hippocampal Dentate Gyrus

In the SGZ, the self-renewing stem cell is thought to be the type 1 cell, a radial glial astrocyte-like cell that expresses nestin, glial fibrillary acidic protein (GFAP), and Sox 2 (Zhao et al. 2008; Encinas et al. 2006). Type 1 cells give rise to type 2 cells, an early neuronal progenitor, which expresses nestin, but not GFAP. Type 2 cells have short processes as compared to the long processes of type 1 cells and can be divided into two types—type 2a cells that lack doublecortin (DCX) and type 2b cells that express DCX (Zhao et al. 2008; Kronenberg et al. 2003). Type 3 cells lack nestin, express DCX, and are immature neurons which migrate a short distance into the granular cell layer where they incorporate as functional neurons in the dentate gyrus and synapse with CA3 targets (Zhao et al. 2008; Kronenberg et al. 2003; Duan et al. 2008; van Praag et al. 2002). In the adult during normal development, these newly generated granular neurons are found typically within the subgranular zone and do not migrate throughout the granular layer. These new neurons from the SGZ of the hippocampal dentate gyrus are thought to contribute to learning and memory.

4.2 The Injured Environment

In many ways, the endogenous neurogenesis that occurs throughout life is merely an extension of the more concentrated and robust development that occurs early on. Both the SVZ and SGZ share characteristics with their developmental counterparts that suggest that many of the mechanisms regulating embryonic development are relevant later in life. This so-called neurogenic niche is comprised of not only neural stem/progenitor cells but a number of highly specialized other cell types including a variety of neurons, astrocytes, pericytes, endothelial cells, and microglia. When injuries such as hypoxia–ischemia or traumatic brain injury occur, this neurogenic niche becomes dramatically altered. Neurons die and release both excitatory and inhibitory neurotransmitters, astrocytes become activated and secrete a variety of both neurotrophic and inflammatory mediators, and microglia also become activated and release a variety of both pro- and anti-inflammatory substances. In addition, the blood–brain barrier becomes disrupted, allowing an influx of circulating factors that otherwise would not normally reach neural cells that mediate both damage and repair (Kohman and Rhodes 2013).

Many substances released by this injury-activated state have been implicated in either enhancing or impairing neurogenesis, and it is likely the balance between these opposing forces that ultimately determines the degree of neurogenesis that occurs in response to injury becomes altered. Although any given injury likely has its own complement of accompanying inflammatory mediators, it has been challenging to study these given the heterogeneous nature of such injuries and that many of the responses are developmentally or species specific and change over time or with relatively subtle differences in genetic makeup. Some classes of these inflammatory mediators known to alter neurogenesis include glucocorticoids, cytokines, chemokines, and prostaglandins.

Activated microglia secrete a variety of proinflammatory cytokines including interleukins such as IL-1β and IL-6 as well as tumor necrosis factor (TNF). Both IL-6 and TNF have been shown to decrease cell proliferation in neurogenic areas and impair neuronal differentiation (Kohman and Rhodes 2013). These effects may be nonspecific and secondary to the generalized inflamed environment. IL-1β, however, has its receptors expressed on hippocampal progenitors, and activation of these receptors directly impairs proliferation and subsequent neurogenesis (Green et al. 2012). Using an elegant genetic approach, Wu et al. inducibly overexpressed IL-1β specifically in neural progenitors and demonstrated its direct effect on neurogenesis by highly attenuating the doublecortin-expressing progenitor population and suggesting that its overexpression shifted cell fate towards a more astrocytic phenotype (Wu et al. 2012). This may in part explain why astrocytes become highly proliferative in response to injury, whereas adjacent neurons and committed precursors are vulnerable.

Microglia do not only secrete proinflammatory cytokines, however, and their net effect on neurogenesis is complicated. Emerging data describe the so-called M2 phenotype for microglia that plays a role in regenerative processes within the brain

(Kohman and Rhodes 2013). These alternatively activated microglia are known to express anti-inflammatory cytokines such as IL-10 as well as growth factor (NGF) and brain-derived neurotrophic factor (BDNF). It appears that microglia activated by traditional lipopolysaccharide (LPS) stimulation take on a proinflammatory phenotype that impairs neurogenesis. When stimulated by anti-inflammatory cytokines such as IL-4, a more neuroprotective phenotype emerges that clearly may be neurogenic under certain circumstances. The balance between these two opposing phenotypes in the setting of injury remains unclear and probably varies by type, duration, and severity of injury.

4.3 Neurogenesis After Injury

Acquired brain injuries are extremely common in both children and adults. In children, traumatic brain injury is the most common cause of death and disability for children over 1 year of age and hypoxic–ischemic brain injury in the neonatal population is also quite common (Koch and Kernie 2011). In adults, while traumatic brain injury and hypoxic–ischemic brain injury still occur, occlusive and hemorrhagic stroke make up a much larger proportion of commonly seen acquired brain injuries (Pendlebury and Rothwell 2009). Neurogenesis in the healthy postnatal/adult brain is altered by all brain injuries including stroke, hypoxia–ischemia, and traumatic brain injury.

4.3.1 Stroke

Ischemic stroke is caused by an abrupt disruption of blood flow to the brain. The most common causes of stroke in humans are due to arterial thrombus/embolus or hemorrhage from the rupture of aberrantly formed arteriovenous malformations. In the presence of absent or decreased cerebral blood flow, ischemia occurs relatively rapidly due to neuronal dependence on glucose for metabolism. Neurons do not store glucose and whether they are capable of using anaerobic metabolites for energy remains controversial, though it is clear that their ability to sustain decreased blood flow is highly diminished compared to other cell types (Sierra et al. 2011; Pellerin et al. 2007). Animal studies that mimic human reductions in cerebral blood flow (CBF) and associated brain injury demonstrate that a 20 % reduction in CBF disrupts protein synthesis, a greater than 50 % reduction results in impaired ATP synthesis, accumulation of lactate and glutamate, shifts in water compartments, and decreased ability for neurons to fire action potentials, and a greater than 80 % decrease in CBF results in electrolyte imbalances and ischemic neuronal death (Hossman 1994). The ability of neurons to adapt to the kind of ischemic changes seen with stroke remains an area of intense focus.

4.3.1.1 Stroke-Induced Neurogenesis in Animal Models

In the most common rodent model of stroke, the middle cerebral artery is transiently occluded for a defined period of time by a filament via the carotid artery, then released, with the extent of damage dependent upon the duration of occlusion (Lindvall and Kokaia 2008). Multiple studies have revealed that endogenous neural precursors stimulate neurogenesis in the SVZ in rodent models of ischemic stroke.

Arvidsson et al. demonstrated that infarction of the rodent striatum, generated by middle cerebral artery occlusion (MCAO) for 2 h, resulted in neural progenitors from the SVZ migrating to the damaged striatum, maturing into neurons that expressed striatal cell markers, and synapsing upon neighboring cells (Arvidsson et al. 2002). They observed neurogenesis in the infarcted striatum after MCAO compared to contralateral or sham-injured striatum through the following experiment: on days 4–6 after stroke versus sham injury, the mice were injected with BrdU, a marker for cell proliferation. At 5 weeks after stroke, there was near-complete loss of NeuN, a neuron-specific marker, in the ipsilateral striatum, but there was a near 31-fold increase in doubly labeled BrdU/NeuN cells of the ipsilateral striatum compared to sham or contralateral striatum. There were also numerous BrdU-labeled cells only in the ischemic tissue likely secondary to gliosis and inflammation. MCAO in this study also resulted in ischemia of the parietal cortex; however, no BrdU-/NeuN-expressing cells were noted in these infarcted areas. They then treated the mice with BrdU and cytosine-B-D-arabinofuranoside (Ara-C), an inhibitor of mitosis in the mouse SVZ, for 12 days after stroke which resulted in a reduction of DCX-expressing cells in the SVZ and striatum and BrdU-/DCX-doubly labeled cells. The stroke-generated DCX-expressing cells showed a more migratory neuron morphology and were distributed along a gradient with a higher density near the SVZ, which tapered towards the striatum. These data suggested that neuroblasts originate from the SVZ and migrate to the striatum. They then injected poststroke mice with BrdU and stained the striatal cells with DARPP-32 (a cell marker for medium-sized spiny neurons, the dominant neuronal cell type in the striatum) and noted that the BrdU-/DARPP-32-expressing cells had morphology consistent with medium-sized spiny neurons in the striatum, which supports the idea that the newly developed neurons replaced damaged striatal neurons.

These findings were reinforced by Yamashita et al. in 2006 in another rodent model of MCAO-induced stroke (Yamashita et al. 2006). After MCAO, they found more DCX-expressing cells in the striatum compared to pre-MCAO striatum suggesting neurogenesis occurring as a result of infarction. They then stereotactically injected the striatum of CAG-CAT-EGFP transgenic mice with the Cre-encoding adenoviral vector, AxCANCre, which would result in the ubiquitous CAG promoter being induced by Cre to express GFP. They found GFP expression in the striatal cells as expected; however, no DCX-expressing cells co-expressed GFP, suggesting a neuroblast origin outside the striatum. Using the Cre–loxP recombination system to label SVZ cells with GFP, after MCAO they found GFP-expressing cells in the ipsilateral striatum, as compared to GFP-expressing cells being restricted to the

SVZ in control animals. This suggests that neuroblasts from the SVZ migrate to the ischemic striatum. To better characterize the migration pattern of the neuroblasts from the SVZ to the striatum, they used electron microscopy and observed chains of neuroblasts elongated parallel to blood vessels, which potentially act as scaffolding upon which the neuroblasts travel. These neuroblasts went on to express the mature neuronal marker NeuN and under electron microscopy had the morphology of mature neurons, synapsed with neighboring cells, and contained presynaptic vesicles. Whether these newly integrated neurons are functional remains unclear.

4.3.1.2 Stroke-Induced Neurogenesis in Humans

Although many animal models demonstrate stroke-induced neurogenesis and migration of immature neurons to peri-infarct areas, human data are much more limited. In brain cortical biopsies of the ischemic penumbra of patients who died from ischemic strokes, evidence for neurogenesis was found by immunohistochemistry. Specimens from those patients with ischemic stroke had increased Ki67 (proliferation related Ki67 antigen, a cell proliferation marker)-expressing cell numbers (compared to cerebral cortex biopsies of patients without brain pathology), and a portion of those cells also expressed doublecortin (DCX, an early neuronal marker). None of the Ki67-expressing cells from the control patients expressed DCX. The morphology of some of the Ki67-/DCX-expressing cells showed a migratory phenotype—an elongated cell with a trailing nucleus (Jin et al. 2006). The origin of these new neurons could not be elucidated, though they were found in perivascular niches, areas known to be neurogenic in rodents. Other studies of postmortem brain biopsies were characterized by increased cell proliferation in the ipsilateral SVZ after ischemic strokes (Marti-Fabregas et al. 2010; Macas et al. 2006).

4.3.2 Traumatic Brain Injury (TBI)

Traumatic brain injury is divided into two phases—primary and secondary injury. Primary injury occurs at the time of impact and includes contusion, hemorrhage, and diffuse axonal injury (Xiong et al. 2013). Secondary injury occurs days to months later and is mediated in part by posttraumatic ischemia, glutamatergic excitotoxicity, and cytotoxic and vasogenic edema and is exacerbated by hypoxemia and hypotension (Kochanek et al. 2011). After moderate to severe traumatic brain injury, the affected brain undergoes irreversible cell necrosis and apoptosis, followed by an inflammatory response including microglial extension to the site of injury and reactive astrocytosis, which has a combination of proinflammatory effects and creation of a protective barrier scar between damaged and healthy tissue (Davalos et al. 2005; Myer et al. 2006). Despite the tremendous and ongoing damage that occurs in reaction to traumatic brain injury, there is typically some degree of recovery and

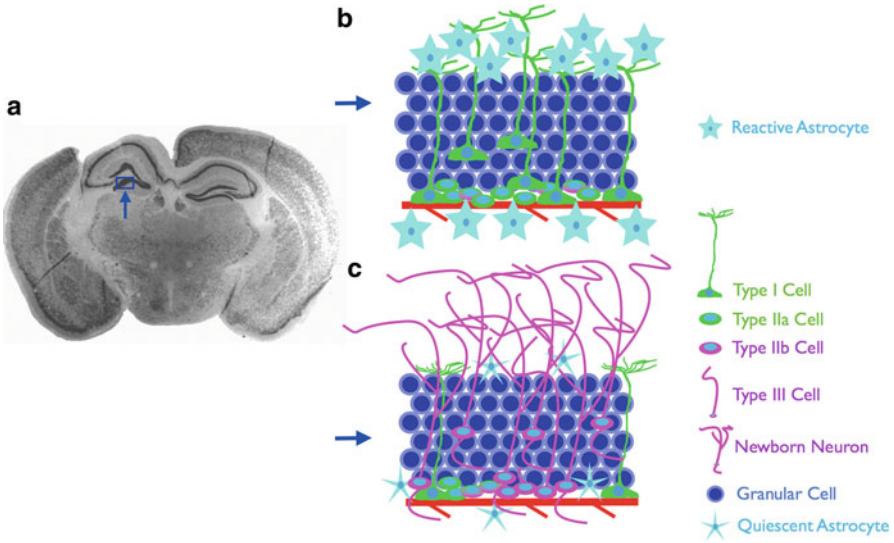

Fig. 4.2 Coronal section highlighting changes to the hippocampus after controlled cortical impact injury in mice (**a**). Three days after injury, astrocytes in the region become thickened and activated while type 1 and type 2 cells become more proliferative and migrate into the granular layer of the dentate gyrus (**b**). One month after injury, the astrocytes are no longer reactive, but there are an abundance of newly born neurons that have integrated themselves into the dentate gyrus (**c**)

self-repair that accompanies these injuries. Emerging data now suggest that neurogenesis from the SVZ and SGZ may contribute new neurons to these injured sites and could potentially underlie some of the observed recovery (Fig. 4.2).

4.3.2.1 TBI-Induced Neurogenesis in Animal Models

Traumatic brain injury is caused by a variety of mechanisms and a single animal model is unable to replicate the spectrum of clinical outcomes. Commonly used rodent models of traumatic injury include fluid percussion injury (FPI), controlled cortical impact (CCI), weight-drop impact acceleration injury, and blast injury (Xiong et al. 2013). Fluid percussion injury is induced by a rapid injection of a fluid pulse into the epidural space, CCI uses an air or electromagnetic piston to directly impact the cortex at a deliberate depth and velocity, weight-drop impact acceleration injury involves dropping a free weight onto exposed dura, and blast injury exposes the rodent to a pressure wave blast inside a chamber. These different models attempt to reproduce the diverse nature of traumatic brain injury in humans. Using these models, it has been observed that traumatic injury induces neurogenesis from the SVZ and SGZ.

Injury-induced neurogenesis was first described when cell proliferation was noted in the SVZ after FPI (Tzeng and Wu 1999). Subsequent research elucidated the

hippocampus as another prominent source of neural precursors for injury-induced neurogenesis. Early observations supporting injury-induced neurogenesis in the dentate gyrus of the hippocampus used CCI as the model for injury in mice, where there was increased BrdU incorporation (marker for cell division) that co-localized with nestin (neuronal precursor marker) within the dentate gyrus of injured mice compared to less BrdU signaling and lack of nestin-expressing cells in sham-injured controls (Kernie et al. 2001). These cells were later studied at 60 days after injury, and some BrdU-expressing cells in the granular layer of the dentate gyrus expressed calbindin-D28 (a marker for mature granular neurons). Similar experiments also found increased BrdU incorporation within the dentate gyrus of injured mice, some cells of which also stained positive for TOAD-65 (an early neuronal marker). When examined over a month after injury, BrdU-expressing cells within the granular cell layer of the dentate gyrus co-localized with calbindin (Dash et al. 2001).

These findings were the first evidence for TBI inducing neurogenesis in the dentate gyrus of the hippocampus. Both studies were limited in definitively demonstrating neurogenesis due to lack of specific neural progenitor cell markers and reliance on nonspecific labeling of dividing cells with BrdU. To overcome this hurdle, a transgenic mouse model was created that expressed GFP under the control of the nestin promoter and its second intron enhancer, which was found to express GFP in neural stem and progenitor cells found in the SVZ of the lateral ventricles and SGZ of the dentate gyrus (Yu et al. 2005). Using this transgenic model, CCI injury was used in conjunction with BrdU before sacrificing the mice at 1, 3, and 7 days after injury (Yu et al. 2008). At 3 days after injury, GFP-expressing progenitors were upregulated in the dentate gyrus ipsilateral to the injury, while DCX-expressing late progenitors were decreased. At 7 days post-injury, the GFP-expressing progenitors continued to increase in the ipsilateral dentate gyrus, and the DCX-expressing cell population reestablished itself within the dentate gyrus. This occurred within a highly proliferative background marked by increased BrdU expression compared to uninjured controls. Therefore, this was consistent with a model of nestin-expressing progenitors being activated by injury, while more mature DCX-expressing neuroblasts remained vulnerable to injury but were able to recover and potentially contribute to stable neurogenesis.

In order to further elucidate whether nestin-expressing early neural progenitors are the actual source of new neurons in the injured dentate gyrus, another transgenic mouse was created that expressed herpes simplex virus thymidine kinase (HSV-TK) and GFP under the control of the nestin promoter and its second intron enhancer. The HSV-TK phosphorylates ganciclovir, converting it to a toxic thymidine analog, which induces cell death in dividing cells. Using these transgenic mice, exposure to ganciclovir at the time of injury resulted in significantly decreased injury-induced neurogenesis in the dentate gyrus compared to unexposed controls (Yu et al. 2008).

4.3.2.2 TBI-Induced Neurogenesis in Humans

Investigations of TBI-induced neurogenesis in human models have been very limited. In a case series of 11 adult patients who suffered from TBI, perilesional

cortical samples were obtained and immunohistologic evidence for neurogenesis was studied and compared to uninjured brain samples (Zheng et al. 2013). Numerous perilesional cells stained positive for markers of cell proliferation Ki67, a human nuclear protein, and MCM2, a minichromosome maintenance protein, which regulates DNA helicase activity, as well as for markers of progenitor or immature neurons (doublecortin, and PSA-NCAM, a polysialylated neural cell adhesion molecule which is involved in cell-to-cell interactions and cell–matrix interactions during differentiation). Compared to controls, cortical tissue from severe TBI patients had increased numbers of Ki67/DCX and Ki67/PSA-NCAM double-positive cells. This suggests that, like in rodent models of TBI, neural progenitors were induced to proliferate after injury, the significance of which remains unknown.

4.3.3 Hypoxic–Ischemic Encephalopathy (HIE)

Hypoxic–ischemic encephalopathy (HIE) arises from generalized hypoperfusion or ischemia of the brain due to conditions such as cardiac arrest, shock, and asphyxiation. Areas of the brain that are vulnerable to such insults are those that lie in vascular watershed regions or have a high metabolic rate and cannot withstand a decrease in oxygen and metabolite delivery, such as the hippocampus and basal ganglia (Petito et al. 1987; Ferriero 2004). Injuries to these areas can result in long-term sequelae such as developmental delays, epilepsy, and cerebral palsy (Ferriero 2004).

4.3.3.1 Hypoxia–Ischemia-Induced Neurogenesis in Animal Models

The Rice–Vannucci model for hypoxic–ischemic encephalopathy (HIE) includes first ligating the common carotid artery, allowing for recovery, then exposing the animal to a hypoxic environment of 8 % oxygen for a given length of time to induce injury in the cortex, hippocampus, striatum, and thalamus ipsilateral to the side of carotid artery ligation (Vannucci and Vannucci 2005). Using this model, hippocampal neurogenesis after HIE was investigated in nestin–GFP transgenic mice, which expressed GFP specifically in neural stem and progenitor cells in young mice (Miles and Kernie 2008). As has been demonstrated in traumatic brain injury, HIE induces proliferation of nestin-expressing hippocampal stem/progenitor cells that incorporate into the dentate gyrus long term and express markers suggesting that they are mature neurons. The functional relevance of this kind of injury-induced neurogenesis remains unknown.

4.3.3.2 HIE-Induced Neurogenesis in Humans

There is one retrospective study of human subjects aged 35–81 years old who were post-HIE from various causes including prolonged resuscitation, acute pulmonary

decompensation, and acute coronary syndrome. These subjects then went on to die at least 24 h after the injury and underwent postmortem brain immunohistochemical staining (Mattiesen et al. 2009). Histologic brain sections were compared with an age-matched control group aged 35–81 years old, who had sudden death from sudden heart failure, asphyxia, and shock but received less than 1.5 h of resuscitation. Brain sections from both groups were stained with proliferating cell nuclear antigen (PCNA), a marker for cell proliferation, TOAD-64/Ulip/CRMP-4 (TUC-4), a marker for immature granule cells during axonal guidance and outgrowth, and calretinin, a marker for young postmitotic neurons. The HIE and control group had no statistical difference in PCNA expression, but the HIE group had significantly increased TUC-4 and calretinin expression supporting upregulation of neurogenesis after hypoxic–ischemic insult in these adult subjects.

4.4 Neurogenesis Aids Recovery

Except after the most severe brain injuries, some spontaneous neurologic recovery inevitably occurs (Campbell et al. 2004). Despite intense investigation, mechanisms underlying this recovery remain largely unknown. Since the first descriptions of ongoing adult mammalian neurogenesis, there has been tremendous speculation suggesting that injury-induced neurogenesis may play a role in the recovery seen after most acquired brain injuries. Many recent studies investigating ablation of neurogenesis after injury support injury-induced neurogenesis as a potential mediator of this recovery.

In a traumatic brain injury model, transgenic mice carrying the nestin–HSV-TK–GFP transgene (described above) underwent unilateral CCI. Animals with both intact and ganciclovir-mediated ablation of neurogenesis underwent numerous behavioral tests including the Morris water maze (MWM), which is specific for hippocampal-based behavior. The MWM is able to test two hippocampal functions—spatial learning and memory (D'Hooge and Deyn 2001). To test spatial learning, a large circular pool is filled with opaque water in which a small platform is hidden beneath the water's surface and external cues are placed outside the pool. During training trials, mice swim within the pool and learn to find the platform. The time taken to find the platform is the endpoint—with progressively shorter times indicating superior spatial learning. The subsequent probe trial removes the small platform and relies upon the mouse's memory of the platform's former location to direct it to the appropriate quadrant of the pool. The percentage of time spent in the target quadrant is the endpoint—longer percentages of time in the target quadrant indicating superior memory.

Nestin–HSV-TK transgenic mice were injured by CCI or sham injured and then treated with ganciclovir (ablating neurogenesis from neural precursors) or inert vehicle. These mice then underwent MWM, at a time (1 month after injury) when newly generated dentate gyrus neurons are known to participate in learning (Ge et al. 2006; Kee et al. 2007). The injured mice treated with ganciclovir took significantly

longer to find the hidden platform and, in the probe trial, spent an equal amount of time in all quadrants as compared to the other three groups who found the hidden platform more quickly and preferred the target quadrant (Blaiss et al. 2011). This demonstrates that injury-induced neurogenesis is required for some aspects of functional recovery following traumatic brain injury. It therefore provides a potential recovery mechanism that can be targeted with drugs that are known to enhance neurogenesis and may improve recovery following traumatic brain injury.

Other groups investigated whether ablation of stroke-induced neurogenesis resulted in poorer neurobehavioral outcomes. Jin et al. created a transgenic mouse that expressed herpes simplex virus thymidine kinase under the control of the doublecortin (DCX) promoter (Jin et al. 2006). Doublecortin is an early neuronal marker and is expressed in the SGZ of the dentate gyrus and the SVZ of the lateral ventricles, areas of ongoing neurogenesis. When treated with ganciclovir, DCX-expressing early neurons were severely depleted in these neuroproliferative regions as compared to vehicle-treated animals. To evaluate the effect of impaired neurogenesis after ischemic stroke, these DCX–HSV-TK mice underwent pretreatment with ganciclovir or vehicle for 14 days and then underwent MCAO-induced infarcts. Subsequent sensorimotor testing including rotarod (time the mouse is able to stay on a rotating rod), limb placing (response of a mouse's forelimb or hind limb to vibrissae stimulation), and elevated body swing (when a mouse is held by its tail, the percentage of time it twists its body to the contralateral side of injury) showed increased deficits in the infarcted, ganciclovir-treated mice as compared to sham-injured or vehicle-treated mice (Schaar et al. 2010; Jin et al. 2010). Further experiments demonstrated that behavioral deficits persisted long term and infarct volumes were larger in progenitor-ablated animals, though it remains unclear whether this is secondary to ablation of injury-induced neurogenesis or due to other support mechanisms elicited from these progenitors (Wang et al. 2012).

4.4.1 Altering Endogenous Neurogenesis

There are now enough studies suggesting that neurogenesis may play a role in recovery following acquired brain injury like TBI and stroke and that manipulating neurogenesis remains a compelling potential therapeutic target. While the exact mechanisms underlying injury-induced neurogenesis remain largely unknown, there are a variety of endogenously produced compounds that are known to influence neurogenesis in development and may play roles in enhancing this response after injury. In addition, there are also many exogenous drugs used for other reasons that are known to augment neurogenesis in animal models. Finally, there are a number of drugs commonly used in the setting of severe acquired brain injury that are known to impair neurogenesis in animal models.

4.4.1.1 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) has been studied in traumatic brain injury and ischemic injury models for its potential therapeutic role in improving clinical outcomes. VEGF induces angiogenesis, is a survival factor for vascular endothelial cells, and may increase vascular permeability (Ferrara et al. 2003). Its expression has been shown to be upregulated by traumatic brain injury and ischemia (Hayashi et al. 1997; Skold et al. 2005) and may contribute to post-injury neurogenesis (Lu et al. 2011). In a rat model of traumatic injury by weight drop, they found that after injury, DCX-/BrdU-expressing cells in the hippocampus (suggesting neurogenesis) had increased VEGF expression levels. After introducing intraventricular VEGF antisense oligonucleotides, they observed a decrease in VEGF expression with an associated decrease in hippocampal neurogenesis (Lu et al. 2011). In an overexpression model of VEGF, transgenic mice were developed that constitutively expressed human VEGF under the control of the neuron-specific enolase promoter, with the highest levels of VEGF expression observed in the hippocampus and cerebral cortex. These mice underwent MCAO and, compared to wild-type controls, had increased BrdU-/DCX-expressing cells, which appeared to migrate from the SVZ to the peri-infarct cortex, and, at 3 weeks following injury, expressed the mature neuronal marker NeuN (Wang et al. 2007). These VEGF-overexpressing transgenic mice also performed better on motor testing and had smaller infarct size.

VEGF treatment acutely after TBI has been observed to increase angiogenesis and neurogenesis, decrease lesion size, and improve neurologic outcomes (Thau-Zuchman et al. 2010). Mice that were subjected to closed head injury with a weight dropped onto a cone placed on the skull were then infused with intraventricular VEGF for 7 days. Afterwards, they showed improved performance on neurobehavioral testing (ten tasks that tested motor ability, balance, and alertness), had increased gliogenesis and neurogenesis by immunohistochemistry, and had decreased lesion volume as compared to vehicle-treated mice. However, it appears that subacute treatment with VEGF after TBI does not have beneficial outcomes, despite there being an increase in angiogenesis. In an experiment where mice underwent closed head injury (weight dropped onto a metallic tip placed on the intact skull) then 7 days post-injury were treated with intraventricular VEGF versus vehicle, there was increased angiogenesis and cell proliferation; however, the majority of the new cells matured into astrocytes and oligodendrocytes. No mature neurons were noted. Functional testing of these mice showed no statistically significant functional benefit with VEGF treatment versus vehicle (Thau-Zuchman et al. 2012). This suggests early treatment with VEGF may have a neuroprotective effect in addition to a neurogenic/angiogenic effect.

4.4.1.2 Erythropoietin

Erythropoietin (Epo) is a cytokine best known for its role in hematopoiesis; however, it has been identified as a mediator of neurogenesis and may offer

neuroprotection after ischemic injury (Dirnagi et al. 2003). It is constitutively expressed in the brain predominantly by astrocytes, but it is also expressed by neurons while the erythropoietin receptor (EpoR) is expressed on neurons, astrocytes, and endothelial cells (Bernaudin et al. 1999). After ischemia, Epo and EpoR are upregulated in the peri-infarct and infarct area (Bernaudin et al. 1999). Experiments with Epo and EpoR knockout mice have been used to elucidate its role in neurogenesis (Tsai et al. 2006). Because the classic EpoR knockout mutation is lethal during embryogenesis, the Cre/loxP system was used to conditionally delete EpoR in glial cells when crossed with hGFAP–Cre transgenic mice, which spares the hematopoietic system and creates phenotypically normal mice. In this model, after MCAO by cauterization and BrdU pulsing, the peri-infarct cortex was stained for DCX/BrdU. Five days after Injury, DCX/BrdU expression was found to be similar and increased compared to pre-stroke levels, in both the transgenic and wild-type animals. However, 7 days after injury, the control mice had a continued increase in the number of DCX-/BrdU-expressing cells in the peri-infarct cortex, while transgenic mice showed no further increase. TUNEL-staining for apoptosis showed no difference in cell numbers in the peri-infarct cortex 5 and 7 days after stroke. These findings suggest that Epo in part mediates neurogenesis and/or migration to the peri-infarct cortex after stroke.

Further characterization of Epo after stroke was performed on neonatal mice that were intraventricularly injected with a GFP-expressing lentivirus that specifically infected neural stem cells and their progeny (Gonzalez et al. 2013). These 7-day-old mice underwent MCAO and received intraperitoneal injections of Epo at 1 and 7 days after injury, and then GFP expression was quantified by immunohistochemistry at 3 days (P10) and 2 weeks (P21) after injury. At 10 days of life, GFP-expressing cells localized around the ventricles and demonstrated increased migration towards the striatum at 3 weeks of age. At both time points, there were a greater number of GFP-/DCX-expressing cells in the injured striatum compared to the vehicle-treated MCAO mice. Interestingly, there were fewer GFP-expressing cells in the SVZ of the Epo-treated MCAO mice at post-injury week 2. The Epo-treated MCAO mice also had increased NeuN-/GFP-expressing cells at 3 weeks of age compared to vehicle-treated MCAO in the striatum. Conflicting with previous data, when they examined CC3 expression, a marker for apoptosis, they noted decreased CC3 expression in the striatum of Epo-treated MCAO mice compared to vehicle-treated controls. They further investigated whether Epo treatment altered cell fate after MCAO-induced stroke by staining for O4 (an oligodendrocyte marker) and GFAP (an astrocyte and early progenitor marker). Epo-treated mice that underwent MCAO had more O4/GFP co-localization and less GFAP/GFP co-localization than the vehicle-treated MCAO controls.

Erythropoietin treatment has also been tested in young adult animal models of traumatic brain injury and demonstrated pleiotropic neuroprotective effects including enhanced neurogenesis (Meng et al. 2011). Mechanisms underlying these neurogenic effects remain unclear and may be due to both direct and indirect effects. Epo is a neuroprotective cytokine that attenuates injury by reducing apoptosis, inhibiting inflammation, enhancing angiogenesis, and enhancing neurogenesis. Its

specific role in both ischemia and TBI remains unknown, but its presence is known to enhance a variety of neuroprotective genes such as brain-derived neurotrophic factor (BDNF) and a number of early growth response genes (Mengozzi et al. 2012).

These findings suggest a role for Epo in promoting poststroke neurogenesis and neuronal migration and influencing cell fate away from astrogliosis. Epo's role in the cell death of these newly generated neurons has yet to be fully elucidated. These studies were performed, however, in neonatal mice whose progenitor pool consists more of oligodendrocyte progenitors, which are probably differentially effected by pro-progenitor factors such as Epo in an age-dependent manner (Wright et al. 2010). One model for Epo's role in stroke is that infarction increases local Epo/EpoR expression, which induces migration of neuroblasts to peri-infarct areas. However, with loss of Epo, continued waves of neuroblasts cannot migrate to the infarcted region and replace ongoing neuronal cell death, thereby impairing recovery in the absence of Epo (Carmichael 2006).

4.4.1.3 Fibroblast Growth Factor

The growing body of knowledge about fibroblast growth factor (FGF) has now identified 22 different FGF genes with three distinct classifications of function—autocrine/paracrine, "hormonelike," and intracellular (Itoh and Ornitz 2008). The classic understanding of FGF is through its autocrine/paracrine actions of binding tyrosine kinase cell surface receptors to initiate intracellular signaling. However, it also exerts "hormonelike" effects by acting over a long distance and binding with less affinity to these receptors. A third set of FGF isoforms are not secreted extracellularly and interact with nuclear or intracellular voltage-gated sodium channels. FGF is intimately involved with embryonal neuronal development, including neural induction of the ectoderm and organization of the brain and peripheral nervous systems (Guillemot and Zimmer 2011).

In early neurogenesis, FGF2 appears to be involved in neuroprotection and repair after neuronal damage (Fagel et al. 2009). Neonatal rats that underwent injury to their motor cortex by direct motor decortication via aspiration through a craniotomy at 10 days of age, then treated with subcutaneous FGF2, were observed to have improved performance in a skilled reaching task. In addition, they were found to have filling of the cavitary lesion caused by the aspiration, as compared to the vehicle-treated controls which had poorer performance and persistence of a cavitary lesion (Monfils et al. 2005). Further studies by this group to elucidate the type and functionality of cells, which filled the lesioned area, demonstrated increased cells expressing DCX and Ki67 (a marker for cell proliferation) in FGF-treated animals when compared to controls. However, both injured groups of animals showed similar numbers of DCX and Ki67 cell numbers in the striatum and SVZ, which was increased from sham-injured animals (Monfils et al. 2006). The ability of the cells filling the lesioned cortex to fire action potentials was measured by EEG. The new cells from the lesioned cortex had the same EEG pattern as unlesioned rat cortex; however, the mean firing rate and peak frequency were decreased. To evaluate

whether this difference was not simply due to an overall difference in signaling, they compared EEGs of a different area of uninjured cortex (prefrontal cortex) and observed the mean firing rate and frequency range between the lesioned and unlesioned rats were the same. Finally, they re-aspirated the newly filled post-injury motor cortex and noted loss of FGF2-mediated improvement on the skilled reach task, which matched the poor functionality of vehicle-treated injured animals. These experiments suggest a role for FGF neurogenesis early in life as a beneficial therapy after injury.

Neonatal neurogenesis remains quite distinct from the more limited neurogenesis that occurs in adults. There also exist compelling data suggesting that FGF2 regulates adult traumatic brain injury-induced neurogenesis within the dentate gyrus. By using knockout mouse models and virally mediated FGF overexpression, Yoshimura et al. demonstrated that FGF-deficient animals had less injury-induced neurogenesis and a smaller dentate gyrus (Yoshimura et al. 2003). In addition, overexpression of FGF using herpes viruses specific for dividing cells increased the number of newly generated neurons in wild-type mice. Together, these data suggest that endogenous FGF influences both developmental and injury-induced neurogenesis and that FGF-mimetics may provide a means for influencing this response therapeutically.

4.4.1.4 Transforming Growth Factor-β

Transforming growth factor-β (TGF-β) is part of a large family of secreted cytokines that also includes activins and bone morphogenetic proteins. It is expressed in a wide variety of tissues and plays a role in cell proliferation, differentiation, and survival (Gomes et al. 2005). Its effects are mediated by binding to "type I" and "type II" receptors, which are heteromeric transmembrane serine/threonine kinase receptor complexes. Binding of TGF-β to the extracellular domain of these receptors induces the type II receptor to phosphorylate the type I receptor which then recruits and activates intracellular Smad proteins to act as regulators of transcription (Feng and Derynck 2005).

TGF-β exists as three isoforms in humans: TGF-β1, TGF-β2, and TGF-β3. In normal conditions, all three are found in the central nervous system; but TGF-β2 and TGF-β3 are expressed more ubiquitously than TGF-β1, which is generally restricted to the choroid plexus (Krieglstein et al. 2011). TGF-βs are involved in various aspects of neuronal development including cell proliferation and survival, differentiation, neurite outgrowth, and synapse formation (Vogel et al. 2010; Fukushima et al. 2007). During development, TGF-β generally arrests cell cycle progression and promotes neuronal differentiation (Vogel et al. 2010).

Ischemic injury appears to induce TGF-β1 expression in the ischemic cortex and injured hippocampus, beyond its typical location in the choroid plexus, and its overall effect is neuroprotective (Klempt et al. 1992; Wang et al. 1995; Zhu et al. 2000). Multiple studies have demonstrated that exogenous overexpression of TGF-β1 is associated with decreased infarct size in MCAO murine models (Henrich-Noack

et al. 1996; Pang et al. 2001) and, conversely, increased infarct size when TGF-β1 activity was antagonized (Ruocco et al. 1999; Dhandapani and Brann 2003). This effect of TGF-β on neurogenesis is somewhat conflicting with some studies showing pro-neurogenic properties, while others show anti-neurogenic effects. In support of its anti-neurogenic effects, Pineda et al. exposed mice to whole-brain irradiation and found a decreased neural stem cell population in the SVZ, which demonstrated decreased proliferative potential when passaged as neurospheres, and an upregulation of TGF-β production by brain endothelial cells (Pineda et al. 2013). When the mice were administered an anti-TGF-β blocking antibody, they observed increased neuroblast production in the SVZ and increased BrdU-/Dcx-double-positive neuroblasts in the olfactory bulb. To further investigate the effect of TGF-β from irradiated brain endothelial cells on neural progenitor cells, they cocultured irradiated blood endothelial cells with SVZ neural progenitor neurospheres and noted an increased phosphorylation of Smad3 in the neurospheres, and subsequent apoptosis, which was reversible with the addition of anti-TGF-β blocking antibody. In this model, TGF-β appears to inhibit neurogenesis and induce apoptosis after injury by radiation.

In support of TGF-β as a promoter of neurogenesis, Mathieu et al. created an overexpression model by stereotactically injecting adenoviral vectors carrying TGF-β or β-galactosidase control into the subventricular zone of adult Wistar rats and observed increased BrdU-expressing cells on the ipsilateral side of the TGF-β injection as compared to controls (Mathieu et al. 2010). Their control animals, however, did show a non-statistically significant trend towards increased neurogenesis on the ipsilateral side to vector injection, which suggests that the injection or adenoviral vector itself may have contributed to the neurogenic response. In an MCAO model of ischemic injury, mice given intranasal TGF-β1 had reduced infarct volume, decreased apoptosis, and increased neurogenesis as evidenced by increased BrdU/NeuN co-stained cells in the ipsilateral SVZ and striatum (Ma et al. 2008).

4.5 Drugs That Are Known to Influence Neurogenesis

In addition to the long list of endogenous compounds known to alter neurogenesis, there are also many drugs indicated for other purposes that are also known to influence neurogenesis. Many of these are used in the setting of brain injury for other reasons in both acute and chronic settings. Acutely following severe ischemic and traumatic brain injuries, sedatives and anticonvulsants are commonly used, and many have been studied in the context of neurogenesis in animal models. These include benzodiazepines, opiates, and barbiturates. In addition, there are also other medications given in the more chronic phase of brain injury that have also been studied for their effects on neurogenesis. These include all classes of antidepressants, anticonvulsants, and statins (HMG-CoA reductase inhibitors). Since injury-induced neurogenesis in humans is currently impossible to evaluate, how these medications might affect neurogenesis-based recovery after ischemic and traumatic brain injuries remains speculative.

4.5.1 Medications That Enhance Neurogenesis

4.5.1.1 Antidepressants

In the neurogenesis hypothesis of depression, it is theorized that decreased adult neurogenesis in the dentate gyrus may contribute to depression, and treatment with serotoninergic medications, which augment neurogenesis, can attenuate depressive symptoms (Jacobs et al. 2000). Multiple studies show the neurogenic effect of various antidepressants (Encinas et al. 2006; Manev et al. 2001). Malberg et al. looked at the effects of fluoxetine (a selective serotonin reuptake inhibitor) and electroconvulsive therapy effects on neurogenesis and found that chronic therapy with fluoxetine (14 days) or electroconvulsive therapy (10 days) increased BrdU-positive cells specifically within the dentate gyrus and that the newly generated cells persisted for at least 28 days. They morphologically appeared like neurons within the dentate gyrus and co-localized with BrdU and the mature neuronal markers NeuN or neuron-specific enolase (NSE) (Malberg et al. 2000). The majority of BrdU-positive cells were neuronal and not glial, and cell proliferation was not noted in the SVZ. Santarelli et al. then proposed that hippocampal neurogenesis was necessary for antidepressants to have a benefit (Santarelli et al. 2003). They used a mouse model of depression, treated them with fluoxetine, and observed increased neurogenesis in the dentate gyrus and improvement in behavioral measures of depression in fluoxetine-exposed mice. They then exposed the depressed mice to low-dose local radiation to the hippocampus to ablate neurogenesis, after which treatment with fluoxetine resulted in no benefit as compared to vehicle-treated irradiated controls. More recent investigation suggests that antidepressant benefit may not be restricted to its effect on neurogenesis, but may also be contribute to neuronal remodeling (Bessa et al. 2009). While the beneficial role of antidepressant-induced neurogenesis remains unclear, it has been definitively established that most classes of antidepressant medications increase hippocampal neurogenesis in rodent models through either direct or indirect means.

4.5.1.2 Statins

Statins are HMG-CoA reductase inhibitors that reduce endogenous cholesterol synthesis and are important clinically as a treatment for hypercholesterolemia (Stancu and Sima 2001). They have also been found to have other beneficial effects beyond lowering cholesterol levels, including improved nitric oxide production from dysfunctional endothelium (Mashimo et al. 2013), reduced platelet aggregation (Tsai et al. 2011), and decreased inflammation (Melo et al. 2013; Tawakol et al. 2013).

Their role in brain injury has recently attracted attention as they have been shown to improve outcomes in intracerebral hemorrhage (Biffi et al. 2011a), stroke (Biffi et al. 2011b), and traumatic brain injury (Mahmood et al. 2009). This beneficial effect may be in part mediated by their putative role in injury-induced neurogenesis.

In a CCI mouse model of traumatic brain injury, it was observed that mice treated with atorvastatin or simvastatin both had improved spatial learning in Morris water maze testing versus saline-treated controls after CCI (Lu et al. 2007). To determine whether neurogenesis may be responsible for this benefit, the dentate gyrus of the injured mice were examined by immunohistochemistry, and there were increased Brdu-/NeuN-co-labeled cells in the ipsilateral dentate gyrus 15 days after injury. Many of the BrdU-positive cells died by day 35; however, the proportion of BrdU-/NeuN-co-labeled cells increased, suggesting improved survival of new neurons and/or continued differentiation of proliferating cells into neurons. The neurogenic effect of statins was also observed in a rat model of intracerebral hemorrhage. Intracerebral hemorrhage was produced by injecting autologous whole blood into the striatum adjacent to the SVZ of rats, which were then treated with atorvastatin, simvastatin, or PBS (Karki et al. 2009). The ipsilateral SVZ had a significantly increased number of BrdU-/DCX-co-labeled cells and the boundary around the hematoma had a significantly increased number of BrdU-/NeuN-co-labeled cells in mice who had received statins versus control, thereby supporting the theory that statins promote neurogenesis after injury. As with antidepressants, it is unclear whether this neurogenic effect is direct or indirect and whether it is at all responsible for the neuroprotective effects attributed to statin treatment.

4.5.2 Medications That Attenuate Neurogenesis

4.5.2.1 Glucocorticoids

Glucocorticoids in humans (primarily cortisol) and in rodents (primarily corticosterone) are secreted in a diurnal rhythm and increase under periods of stress (Dickmeis 2009). Hippocampal neurons express two types of glucocorticoid receptors, the type I mineralocorticoid receptor and the type II glucocorticoid receptor, which when bound, mediate neuronal excitability and inhibit long-term potentiation (Morimoto et al. 1996; Pavlides et al. 1996; Joels et al. 2009). The hypothalamic–pituitary–adrenal axis is activated in response to injury and results in the systemic release of glucocorticoids. In moderate doses, mouse corticosterone appears to enhance neurogenesis, while at higher doses it clearly inhibits it (Wolf et al. 2009). In instances of acute increases of corticosterone that are seen following acquired injuries such as TBI, increases in neurogenesis have recently been attributed to the direct effects of corticosterone on astrocytes, which secrete FGF2, thereby acting as an enhancer of neurogenesis (Kirby et al. 2013). Glucocorticoids, however, are known to have ubiquitous effects, and recent data suggest that they function epigenetically in dentate gyrus progenitors to affect methylation states, thereby influencing neurogenesis, which could explain why varying amounts and duration of glucocorticoid exposure can affect neurogenesis in opposing ways (Yang et al. 2012).

Studies on the effects of glucocorticoids in murine models have revealed inhibitory effects on adult neurogenesis. In rats who received subcutaneous injections of corticosterone to elevate blood levels, there was a significant decrease in newly born cells in the hilus of the dentate gyrus (Cameron and Gould 1994). These rats were then adrenalectomized in order to eliminate adrenal steroids, which resulted in an increased number of [^{3}H]-thymidine (a marker for cell proliferation) and NSE-co-labeled cells indicating increased newly born neurons in the dentate gyrus. This effect, however, was not reversed with the addition of corticosterone (Cameron and Gould 1994). Further supporting this observation were experiments by Brummelte and Galea who injected high-dose corticosterone intraperitoneally in rats and noted a decreased number of BrdU- and DCX-co-localized cells after 7 days of corticosterone and a decreased number of BrdU- and NeuN-expressing neurons after 28 days of corticosterone treatment (Brummelte and Galea 2010). They also subjected these rats to adrenalectomy to remove endogenous adrenal steroid exposure and observed an increase in the total number of new neurons in the dentate gyrus as compared to non-adrenalectomized animals (Brummelte and Galea 2010). Together, these studies suggest a role for endogenous corticosteroids in the attenuation of the neurogenic response.

4.5.2.2 Benzodiazepines

Benzodiazepines are a class of medications that act mainly on the GABA(A) receptor to potentiate its inhibitory CNS effects, although the exact mechanism is still somewhat unclear (Campo-Soria et al. 2006). There are conflicting data regarding the effect of benzodiazepines on neuronal apoptosis, and its role in adult neurogenesis has not been well characterized, although data suggest it inhibits neurogenesis. In rats treated with 20 mg/kg of diazepam and then injected with BrdU, there was a decreased density of BrdU-positive cells in the brains of rats treated with diazepam as compared to saline control (Stefovska et al. 2008). A study exploring the specific effect of benzodiazepines on neuronal function showed that exposure of hippocampal cell cultures to midazolam decreased the amplitude and frequency of intracellular Ca^{2+} oscillations, which altered regulation of neuronal development and differentiation. The perturbation of Ca^{2+} oscillations resulted in reduced synapsin expression, a nerve terminal-specific protein, which suggested decreased neuronal plasticity after benzodiazepine exposure (Sinner et al. 2011).

GABA and GABA(A) receptors and their role in neurogenesis and brain injury have also been investigated. The role of GABA in adult neurogenesis parallels its role in embryonic neurogenesis as described by Esposito et al., who injected dividing neural progenitor cells in the dentate gyrus of mice with a retroviral vector expressing GFP, and observed the interplay of GABA with the proliferation of neural progenitors (Esposito et al. 2005). They observed that new neuronal maturation followed a sequential pattern of silence (no conductance), while the neural progenitor was in the SGZ. The progenitors then developed slowly conducting GABA afferents as they migrated into the inner molecular layer. This was followed by

glutamatergic afferents as they continued to mature and extend spiny dendrites to the outer molecular layer, and finally fast GABA afferents were noted as they completed maturation (Esposito et al. 2005). These observations suggest a role of GABA as a mediator of adult neurogenesis. Regarding GABA's relationship with brain injury, in a lateral FPI model of traumatic brain injury, it was observed that there was downregulation of GABA(A) receptor subunits α1, α4, γ2, and σ which might be mediated through activation of the JaK/STAT and Egr3 pathways (Raible et al. 2012). As GABA(A) receptors are the target for benzodiazepines, one could speculate that these experiments give insight into the effects of benzodiazepines on injury-induced neurogenesis; however, the exact mechanism underlying the role of benzodiazepines on neurogenesis remains unclear.

4.5.2.3 Antiepileptics

Seizures are marked by abnormal electrical activity in the brain, which can lead to aberrant neurogenesis in the hippocampus (Parent and Murphy 2008). Antiepileptic drugs (AEDs) include a broad range of medications used to control seizures, and their mechanisms of action vary from drug to drug. Due to this variability, drugs have been studied individually for their specific effects on neurogenesis. These studies have revealed that, as a broad class, antiepileptics tend to inhibit postnatal neurogenesis via unclear mechanisms. Chen et al. exposed rat pups from P7 to P34 to phenobarbital, clonazepam, carbamazepine, valproic acid, or topiramate to characterize the effect chronic AED exposure had on hippocampal neurogenesis (Chen et al. 2009). On the last day of AED therapy, the rats received intraperitoneal BrdU, then after 24 h the dentate gyrus was examined for BrdU expression and co-localization with neuronal markers. They observed that BrdU-expressing cells were far fewer and had poorer survival in phenobarbital- and clonazepam-exposed rats; however, these rats had a higher percentage of BrdU-expressing cells co-localize with DCX. They then withdrew all AEDs for 28 days and observed a notable decrease in NeuN-expressing neurons in the phenobarbital- and clonazepam-treated animals compared to the animals treated with other AEDs. The findings in this study clinically correlate with cognitive deficits being associated with phenobarbital and benzodiazepine use in pediatric patients and suggest a role for disrupted neurogenesis as a possible mediator for this cognitive deficit (Ijff and Aldenkamp 2013).

4.5.2.4 Opiates

Opioids are a class of drugs generally used to achieve analgesia by acting on three opioid receptors: σ, μ, and κ (Bovill 1997). These receptors are G-protein-coupled receptors, which via their intracellular signaling induce calcium and potassium channels to hyperpolarize and inhibit membrane excitability (Waldhoer et al. 2004).

Exposure to opiates has been shown in animal models to inhibit adult neurogenesis. Eisch et al. exposed rats to 5 days of morphine and then injected BrdU on day

6. Their brains were examined either on day 6 or day 28 (Eisch et al. 2000). In the day 6 brains, they observed decreased BrdU-expressing cells in the dentate gyrus of these "chronically" morphine-treated rats compared to controls, and the decreased cell proliferation was attenuated by coadministration of morphine with naltrexone (an opioid receptor antagonist). The day 28 brains showed a persistent decrease in BrdU-expressing cells in the dentate gyrus of morphine-treated mice compared to controls, and the vast majority of cells were mature neurons. Rats that received a single dose of morphine did not show inhibition of neurogenesis. More recent studies further clarified the manner by which morphine works to inhibit neurogenesis. Arguello et al. found a decrease in cell proliferation in the dentate gyrus after morphine administration and observed proliferating cells in the S phase being more sensitive to morphine's inhibitory effects as compared to other cycling cells (Arguello et al. 2008). They also speculated that morphine affected progression of neuronal maturation based upon their observation that following morphine exposure, there was an increased percentage of BrdU-expressing cells that were type 2a progenitors and a decreased number of BrdU-expressing immature neurons as compared to controls. Together, these findings support a role for morphine in inhibiting hippocampal neurogenesis.

4.6 The Role of Chronic or Repetitive Brain Injury on Neurogenesis

Most of what is known regarding hippocampal neurogenesis as outlined in the preceding sections is based on acute and severe brain injury. It is becoming increasingly apparent, however, that some of the most devastating brain injuries occur in response to repetitive injuries associated with recurrent nearby explosions experienced by military personnel or repeated direct brain trauma seen in more violent sports such as American football (Goldstein et al. 2012). Both in humans and animal models, these types of injuries cause persistent activation of microglia and astrocytes as well as accumulation of phosphorylated tau. These histopathologic findings are the basis for the diagnosis of chronic traumatic encephalopathy (CTE) whose features are oftentimes most prominent in the hippocampus (Goldstein et al. 2012).

4.6.1 Chronic Histopathologic Changes in the Hippocampus

Although it remains unclear how some of the chronic changes associated with CTE in the hippocampus affect neurogenesis, emerging evidence suggests that other histopathologic changes seen in addition to activated glia, microglia, and phosphorylation of tau occur that may affect the regenerative response. A subset of individuals

exposed to TBI, either acutely or chronically, may develop a seizure disorder. Although neurogenesis in the context of seizures is examined in great detail in Chap. 10, the permanent changes in the hippocampus such as mossy fiber sprouting and aberrant hilar neurogenesis may influence the proliferative response of the hippocampus following recurrent injury (Hunt et al. 2009). Apart from the generation of new mossy fibers seen in the context of injury-induced epilepsy, there are also vascular changes that occur following TBI that, like mossy fiber proliferation, results in vasculogenesis in the injured hippocampus, and given that the progenitor population is so closely associated with the vasculature, this may affect how neurogenesis occurs in more chronic injuries (Fig. 4.3) (Hayward et al. 2010).

4.6.2 Hippocampal Progenitors Diminish Over Time

The regenerative capacity of the dentate gyrus may diminish over time, and this attenuation may be accelerated in the context of repeated injuries. It was recently demonstrated that recurrent brain injury in mice results in a diminished hippocampal neurogenic response (Gilley and Kernie 2011). The mechanisms underlying this response remain unclear though one intriguing possibility involves ApoE, an important regulator of cholesterol metabolism and an independent predictor of outcome following TBI. ApoE is known to be a negative regulator of neurogenesis in the developing hippocampus, and when it is absent in mouse models, the hippocampal progenitor pool becomes prematurely exhausted and therefore may affect how injury-induced neurogenesis occurs, particularly in chronic injury conditions such as CTE (Yang et al. 2011).

4.7 Conclusions

Ongoing neurogenesis demonstrates structural plasticity of the adult brain, which appears to be an important factor in its ability to recover from acquired injury. Observations in experimental animal models suggest that injury activates neural progenitors from the SVZ and SGZ to proliferate and develop into stable neurons, which contribute to functional recovery. This neurogenesis is in turn influenced by various endogenous factors such as VEGF, Epo, and FGF. In addition, many classes of medications given for a variety of disorders are known to enhance neurogenesis. Therefore, there are many potential candidates for enhancing recovery following injury by directly or indirectly stimulating the progenitor population. This ever-growing knowledge about both endogenous and exogenous factors that influence and promote neuronal proliferation opens the opportunity to ultimately determine whether manipulating neurogenesis has real therapeutic potential following injury.

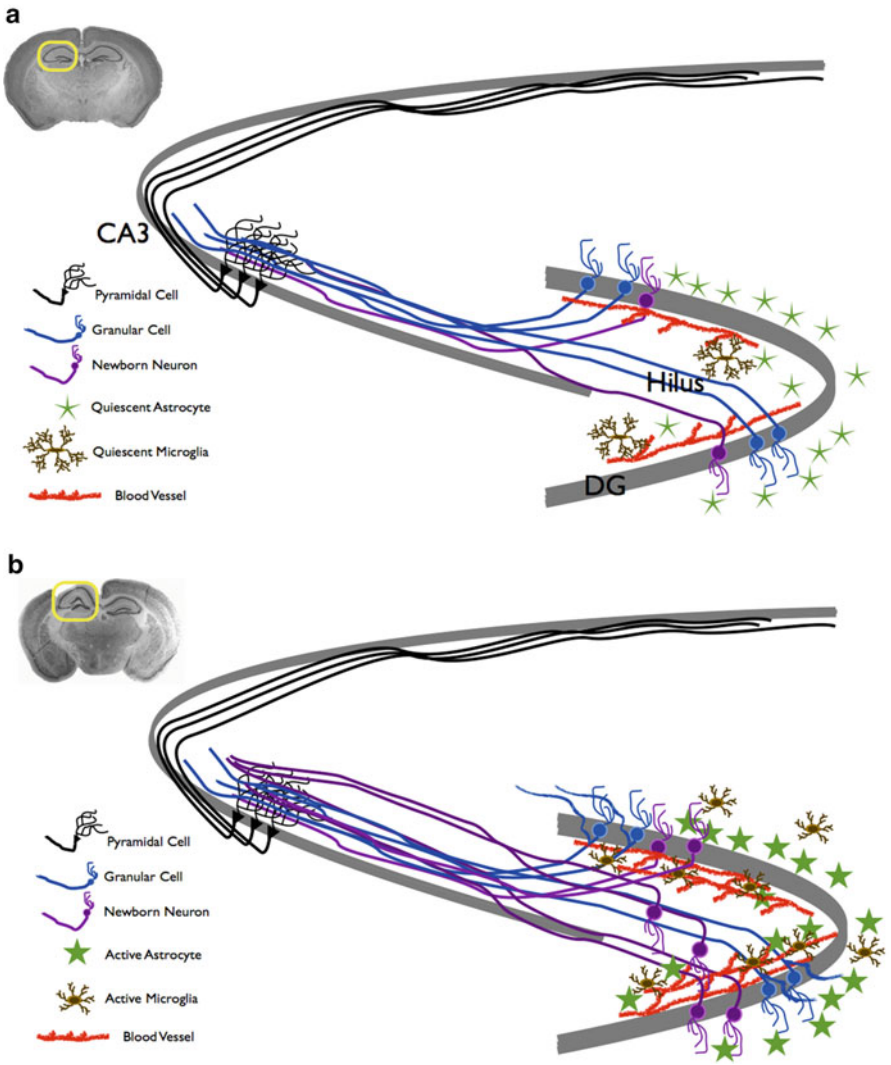

Fig. 4.3 Hippocampal cellular reorganization that occurs long-term following severe or chronic TBI. Compared to the uninjured hippocampus (**a**), the chronically injured hippocampus (**b**) shows increased aberrant neurogenesis both within in the neuronal layers of the hippocampus as well as the hilus, persistent activation of astrocytes and microglia, and neovascularization

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Chapter 5
Role of Adult Neurogenesis in Seizure-Induced Hippocampal Remodeling and Epilepsy

Alison L. Althaus and Jack M. Parent

Abbreviations

BDNF	Brain-derived neurotrophic factor
DCX	Doublecortin
DGC	Dentate granule cell
FGF-2	Fibroblast growth factor 2
GCL	Granule cell layer
HBD	Hilar basal dendrite
HEGC	Hilar ectopic granule cell
IML	Inner molecular layer
KA	Kainic acid
MFS	Mossy fiber sprouting
MML	Middle molecular layer
NSC	Neural stem cell
OML	Outer molecular layer
SE	Status epilepticus
SGZ	Subgranular zone
TLE	Temporal lobe epilepsy
VEGF	Vascular endothelial growth factor

A.L. Althaus (✉) • J.M. Parent (✉)
Department of Neurology and Neuroscience Graduate Program,
University of Michigan, 5021 BSRB, 109 Zina Pitcher Place,
Ann Arbor, MI 48109, USA
e-mail: althausa@umich.edu; parent@umich.edu

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3_5, © Springer Science+Business Media New York 2014

5.1 Introduction

Epilepsy is a complex and diverse neurological disorder characterized by spontaneous recurrent seizures. Of the large variety of epilepsy syndromes, temporal lobe epilepsy (TLE) is the most common in adults. It is also one of the most intractable: in more than 30 % of persons with TLE, seizure activity is not controlled by pharmacotherapy (Engel and Pedley 1998). In many TLE patients, removal of the seizure focus, which includes the hippocampus and other anteromedial temporal lobe structures, alleviates seizures. These resected tissues, along with postmortem tissues, often display hippocampal sclerosis, which includes pyramidal cell death, astrogliosis, and structural reorganization within the dentate gyrus (Engel and Pedley 1998). Pathology in the dentate gyrus is of particular relevance to those interested in adult neurogenesis, because the dentate is a well-established region of ongoing neurogenesis in the adult human brain (Eriksson et al. 1998).

The causes of TLE are largely unknown and probably vary across patients. In many cases, the onset of spontaneous seizures is preceded by an initial precipitating injury, which is believed to play a causal role in the development of epilepsy. Precipitating injuries are different from patient to patient, ranging from prolonged febrile seizures, to nervous system infections like bacterial meningitis, to traumatic brain injury and others. However, most people who experience these insults will not go on to develop epilepsy (Harvey et al. 1997). For those that do, the latent period (time between the injury and onset of seizures) is highly variable. All of these factors contribute to the difficulty of understanding the process of epileptogenesis, which involves cellular and molecular changes leading to the generation of spontaneous recurrent seizures.

Because surgical tissue from patients with epilepsy is typically obtained during late disease stages, this tissue has limited utility for understanding epileptogenesis. Therefore, animal models have been developed to study this process. The most common models use status epilepticus (SE), a prolonged period of continuous seizures, as the initial precipitating injury to produce spontaneous seizures in rodents. In these models, SE is induced by chemoconvulsants (usually pilocarpine or kainic acid) or electrical stimulation, and then spontaneous seizures develop after a latent period lasting days to weeks. Structural changes in the hippocampi of these animals arise within days of SE, and after weeks to months, much of the hippocampal histopathology resembles that seen in hippocampal tissue from human TLE patients (Buckmaster 2004). Within the dentate gyrus, this histopathology includes hilar and pyramidal cell death, dentate granule cell (DGC) layer dispersion, sprouting of mossy fibers, and ectopic locations of DGC bodies (Dudek and Sutula 2007; Houser 1992).

In the late 1990s two reports showed that seizure activity potently stimulates dentate gyrus neural stem cell (NSC) proliferation in rodent models of TLE (Bengzon et al. 1997; Parent et al. 1997). Over the past 15 years, this finding has been replicated in nearly every rodent TLE model (Scharfman and McCloskey 2009), and understanding the relationship between altered NSC behavior and epileptogenesis has become a large focus of the field. The data that emerged have led

to a paradigm shift in our understanding of epilepsy-related pathology and strong interest in the potential for targeting NSCs for therapeutic intervention in TLE. In this chapter we will cover the prominent findings related to how seizures affect proliferation, survival, and development of DGC progenitors and their progeny. Although the impact of epileptic insults on DGC development and its subsequent contribution to epilepsy progression remains uncertain, we will describe recent work aimed at understanding these processes and offer suggestions for future directions. Recent data suggest subtle, but important, differences between levels and functionality of neurogenesis between rats and mice (Snyder et al. 2009). We will present pertinent data both from species and from a variety of models, while trying to highlight instances where discrepancies in the reported data may relate to the use of different species or models.

5.2 Cell Proliferation

Under basal laboratory conditions, about 9,000 new DGCs are generated daily in the young adult rat (Cameron and McKay 2001). Typically, 25–40 % of newly generated DGCs survive and functionally integrate into the dentate gyrus network (Kempermann et al. 2003; Tashiro et al. 2007). However, the number of cells generated and the proportion that survive into maturity are dynamically regulated and can be influenced by external stimuli at many different stages. For example, traumatic brain injury appears to stimulate cell division of a subset of dentate NSCs (Yu et al. 2008). Environmental enrichment tends to affect survival of newly generated DGCs more than the number of cells that are born (Kempermann et al. 1997; van Praag et al. 1999). Seizures, however, probably influence adult dentate gyrus neurogenesis at all stages in the neurogenic process, including proliferation of multiple NSC types, survival, maturation, and integration of adult-generated DGCs.

5.2.1 Response of Progenitor Populations to Seizures

The population of NSCs in the adult dentate gyrus is complex and heterogeneous, and this heterogeneity is not well understood. This has recently been comprehensively reviewed (Duan et al. 2008; Faigle and Song 2013). Determining the responses of the different subsets of NSCs to seizure activity requires a nuanced approach to identifying NSC subpopulations that has not yet been undertaken in TLE models. However, many studies have distinguished the responses of at least two different progenitor populations to seizure activity. Radial glia-like stem cells are multipotent NSCs which are typically quiescent but participate in the early proliferative response to SE in the rodent dentate gyrus (Fig. 5.1) (Huttmann et al. 2003; Kronenberg et al. 2003; Lugert et al. 2010; Seri et al. 2004; Suh et al. 2007). Doublecortin (DCX)-expressing neural progenitors substantially increase in number several days after the

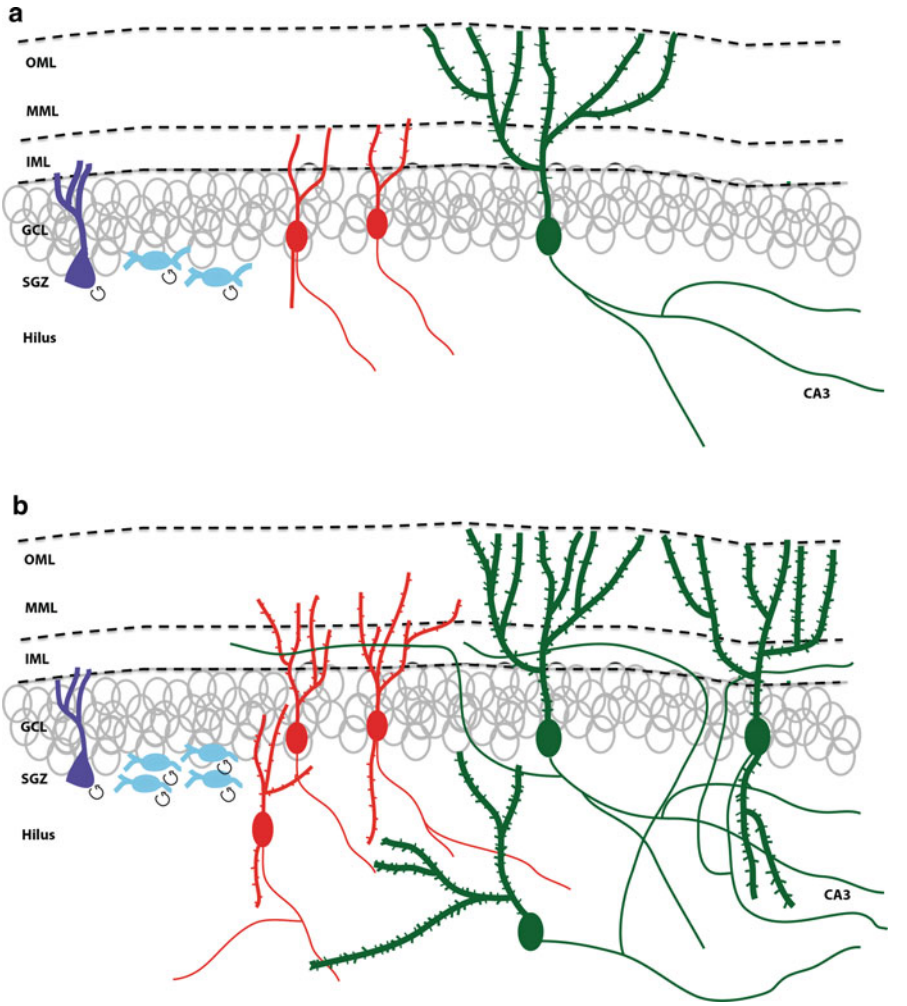

Fig. 5.1 Schematic images of NSC and DGC organization within the intact (**a**) or representative epileptic (**b**) adult dentate gyrus. *Purple* structure represents a radial glia-like stem cell, *blue* represents amplifying neural progenitors, *red* represents immature DGCs, and *green* represents mature DGCs. OML = outer molecular layer, MML = middle molecular layer, IML = inner molecular layer, GCL = granule cell layer, SGZ = subgranular zone. (**a**) In an intact animal, the radial glia-like stem cell and amplifying progenitors are present in the SGZ. Radial glia-like cells are relatively quiescent and unlikely to be dividing at any given time. Amplifying progenitors are a relatively proliferative population and more likely to be dividing. Immature neurons are beginning to develop processes and may have a transient hilar basal dendrite, but it does not receive synaptic input. Mature DGCs, including those born in adulthood, have fully arborized dendritic trees that extend to the outer molecular layer and mossy fiber axons that branch in the hilus and extend out to area CA3. (**b**). In an animal with epilepsy, radial glia-like stem cells may be activated early after SE in some models, but still generally remain quiescent. Amplifying progenitors, however, are commonly upregulated by seizure activity in many models. Immature neurons develop more rapidly, showing longer processes and more dendritic spines. Some immature neurons migrate ectopically into the hilus, where they remain throughout maturity. Once the cells mature, they may retain hilar basal dendrites, be ectopically located in the hilus, or sprout mossy fiber axons into the inner molecular layer

initial SE episode and, depending on the severity and duration of SE, continue to accumulate for up to 4 weeks afterward (Jessberger et al. 2005; Jessberger et al. 2007b; Parent et al. 1997, 1999). DCX-expressing cells are committed to a neuronal fate (Francis et al. 1999); thus, most of the proliferating progenitor cells generate new neurons after SE. The survival of these post-SE-generated cells, however, is closely tied to seizure severity and duration (Mohapel et al. 2004). Interestingly, proliferation can be dramatically increased even with a mild seizure stimulus. In fact a single, discrete electrical stimulation-induced discharge is sufficient to increase neurogenesis several weeks later (Bengzon et al. 1997).

5.2.2 *Effects of Chronic Seizures and Aging*

Fewer studies have focused on neurogenesis in the chronic phase of epilepsy. Although it is well known that basal levels of neurogenesis decline with age in the rodent dentate gyrus (Kuhn et al. 1996), it is unclear how chronic epilepsy influences this age-related decline. Recent work suggests that 5 months after chemoconvulsant-induced SE, rats have dramatically reduced basal neurogenesis compared to same-aged controls (Hattiangady et al. 2004). Although the mechanism for this reduction is unknown, it may result from impaired NSC function in the epileptic brain, or perhaps from a reduction in the available "NSC pool." However, others have found that 6 months after electrically induced SE, epileptic animals do not show a decrease in basal neurogenesis compared to age-matched controls (Bonde et al. 2006), highlighting the variability between models and the importance of understanding how different models relate to human disease.

The age of an animal at the onset of SE is also an important factor in the NSC response. Typically, epilepsy is induced in juvenile/young adult rodents (1–4 months of age). Animals within this age range have equivalent levels of post-SE neurogenesis, despite slight decreases in basal neurogenesis (Gray et al. 2002). However, 24-month-old animals, and in some experiments even those as young as 12 months, do not show increased neurogenesis in response to an epileptogenic insult (Hattiangady and Shetty 2008; Rao et al. 2008). Importantly, this finding is not the result of a general unresponsiveness of NSCs in aged animals, since increased neurogenesis in response to voluntary exercise is maintained in senescent animals (van Praag et al. 2005). Instead, the differential response suggests a specific reduction in NSC activation by seizures in the aged brain.

5.2.3 *Possible Mechanisms of Proliferative Response to Seizures*

Clues as to why the NSC response differs among models or between the young and old brain may come from a better understanding of the molecular mechanisms that lead to enhanced proliferation after an epileptogenic insult. Part of the difficulty in

studying this phenomenon is the fact that molecular mechanisms mediating adult neurogenesis in the intact dentate gyrus are not completely understood. Recent evidence suggests that many of the same processes involved in neurogenesis during brain development also regulate adult neurogenesis (Faigle and Song 2013). Some of these molecular pathways are also stimulated by SE (Elliott and Lowenstein 2004). For example, data indicate that SE alters Notch1 and Sonic hedgehog signaling in a manner that would promote cell proliferation (Banerjee et al. 2005; Sibbe et al. 2012). Trophic factors such as brain-derived neurotrophic factor (BDNF), fibroblast growth factor 2 (FGF-2), and vascular endothelial growth factor (VEGF) are increased in hippocampal tissue after SE (Gall 1993; Isackson et al. 1991; Newton et al. 2003; Warner-Schmidt and Duman 2007) and are also known regulators of adult neurogenesis (Faigle and Song 2013). An important consideration, however, is that changes observed in these signaling cascades after epileptic insults may be incidental to the fact that proliferation has been stimulated, rather than the direct mechanism of stimulation.

Importantly, neuronal activity itself can modulate NSC proliferation (Deisseroth et al. 2004). This effect is mediated through calcium channels, NMDA receptors, and possibly GABA-A receptors expressed by NSCs (Deisseroth et al. 2004; Tozuka et al. 2005). In the dentate gyrus, zinc is an additional potential link between neural activity and NSC proliferation. Zinc is normally released from DGC axon terminals, and zinc chelation after SE reduces NSC proliferation (Kim et al. 2012). Activity-dependent epigenetic modifications are also associated with altered NSC proliferation after single electroconvulsive seizures or chemoconvulsant-induced SE (Jessberger et al. 2007a; Ma et al. 2009), although in one of these settings, neurogenesis is modulated indirectly through epigenetic changes in mature DGCs (Ma et al. 2009). The myriad of diverse signals that have been reported to mediate NSC proliferation after discrete seizures or SE cannot be explained by studies using different epilepsy models because the same models are often used in a number of different studies. Rather, because seizures elicit a variety of changes in neural tissue, it is likely that within any given model, many different signals converge to produce a robust seizure-induced neurogenic response.

5.3 Cell Maturation and Integration

The percentage of adult-generated DGCs that survive the initial activity-dependent selection process is highly variable, even under baseline conditions. Some studies have reported as much as 75 % survival, others as little as 30 %, likely reflective of species and strain differences (Dayer et al. 2003; Kempermann et al. 2003; Snyder et al. 2009; Tashiro et al. 2007). In SE models, the percentage of post-SE-generated cells that survive and mature seems to be, in part, a function of the severity of SE (Mohapel et al. 2004) and a consequence of inflammation in the epileptic hippocampus (Ekdahl et al. 2003). The environmental changes that take place during epileptogenesis, as well as in the setting of chronic epilepsy, affect the maturation

and integration of the surviving, seizure-generated DGCs. Moreover, whether these changes influence DGC progenitors to develop in a pro- or antiepileptic fashion may itself be a function of model severity.

5.3.1 Seizures Affect the Rate of DGC Maturation

In intact animals, adult-born DGCs progress through distinct maturation stages over a period of 3–4 months, after which they are fully integrated into the preexisting network and are indistinguishable from perinatally generated DGCs (Esposito et al. 2005; Laplagne et al. 2006; Piatti et al. 2006; Toni et al. 2008; van Praag et al. 2002; Zhao et al. 2006). Each stage of maturation is regulated by both intrinsic and extrinsic mechanisms. GABA plays an important role at many stages (Ge et al. 2007). Because of the high internal chloride concentration of adult neural progenitors and immature neurons, GABA depolarizes the membrane and elicits an excitatory response that is necessary for proper development (Ge et al. 2006; Overstreet Wadiche et al. 2005; Tozuka et al. 2005). Since the developing cells do not respond to glutamatergic inputs until they are about 2 weeks postmitotic (Piatti et al. 2006), tonic and synaptic GABA inputs drive much of the early activity-related development. This effect is, in part, mediated by the basic helix-loop-helix transcription factor NeuroD1, which is activated by GABA-driven activity and is required for survival and maturation of DGCs (Gao et al. 2009; Tozuka et al. 2005). Many SE models show profound changes to GABAergic activity in the dentate gyrus, due to the death of inhibitory interneurons and changes in the structure and function of the remaining interneurons (Dudek and Sutula 2007; Thind et al. 2010; Zhang et al. 2009). A direct relationship between altered GABA signaling and altered neurogenesis has not been explored in the context of TLE models, but this is a promising area for future research. Changes in network activity and in levels of growth factor expression also affect the rate of maturation and integration of adult-born DGCs (Piatti et al. 2011; Waterhouse et al. 2012). Not surprisingly, seizures also strongly affect the rate of DGC development. Under baseline conditions, DGC dendrites do not reach the outer molecular layer until about 21 days after birth (Toni et al. 2007). However, some DGCs born after or near the time of SE develop extensive dendritic arbors almost a week sooner and receive excitatory inputs well before their counterparts in control brains (Overstreet-Wadiche et al. 2006).

5.3.2 Aberrant DGC Migration in Epilepsy

In addition to speeding up maturation and integration, alterations in the local environment of the dentate gyrus after SE lead to abnormal DGC morphological features and physiology. Adult-born DGCs are generated in the subgranular zone

(SGZ), between the granule cell layer (GCL) and the hilus. Normally, as they mature, they migrate into the GCL where their dendrites receive excitatory inputs from perforant path fibers. After SE, a subset of the newly generated DGCs migrates aberrantly into the hilus where they are innervated by mossy fiber axons (Jessberger et al. 2007b; Kron et al. 2010; Parent et al. 2006; Pierce et al. 2005). Newborn DGCs continue to migrate ectopically even in chronic epilepsy after the level of neurogenesis has returned to baseline (Bonde et al. 2006), suggesting that permanent changes to the epileptic network underlie the aberrant migration. Although the causes of aberrant migration are not well understood, reelin, a migratory signal that is involved in embryonic development, is one interesting candidate. Reelin signaling is important for proper migration of early postnatal and adult-born neurons in the dentate gyrus (Gong et al. 2007; Teixeira et al. 2012) and is potently disrupted by SE (Gong et al. 2007). Importantly, the loss of reelin signaling within individual DGCs in an otherwise normal animal is sufficient to induce ectopic migration of the affected cells (Teixeira et al. 2012).

Loss of reelin signaling has also been linked to aberrant locations of mature DGCs in the intra-hippocampal kainate model of SE (Heinrich et al. 2006). In this model, the chemoconvulsant kainic acid (KA) is delivered directly into the hippocampus of one hemisphere. This induces SE and robust cellular pathology, including dispersion of the granule cell body layer in the ipsilateral (injected) hippocampus. This dispersion, which can be observed rapidly following the KA injection, is not associated with an increase in neurogenesis (Fahrner et al. 2007), likely due to disruption of the dentate NSC niche from severe injury. Thus, work from different animal models indicates that dispersion of the normally compact granule cell body layer may result both from acute changes to the structure of mature DGCs and from chronic changes that impair migration of developing DGCs.

Due to their aberrant location and inputs, hilar ectopic granule cells (HEGCs) are thought to play an important role in the formation of a recurrent excitatory network after SE (Parent and Lowenstein 2002; Scharfman and Gray 2007). In addition to being innervated by mossy fiber axons, HEGCs send their axon collaterals to the molecular layer to form aberrant synapses onto DGC apical dendrites (Scharfman et al. 2000). Functionally, they receive more excitatory inputs than DGCs located in the GCL (Zhan et al. 2010; Zhang et al. 2012), and they become partially synchronized with pyramidal cells in area CA3 (Scharfman et al. 2000). However, HEGCs are probably not the only drivers of aberrant excitatory activity in the epileptic dentate gyrus. Many, perhaps most, other DGCs participate to some extent in the formation of the abnormal epileptic network. A major difficulty in defining the net effects of altered DGC neurogenesis on epileptogenesis, however, is that the DGCs in the granule cell layer, even those only generated after SE, are likely to be a heterogeneous population, with some contributing to excess excitability, others having a more neutral response, and still others perhaps playing a compensatory role by developing reduced excitability.

5.3.3 *Aberrant Dendritic Morphology in Epilepsy*

One subpopulation that is believed to contribute to the recurrent network is DGCs with hilar basal dendrites (HBDs) (Dashtipour et al. 2003; Ribak et al. 2000; Shapiro et al. 2005; Shapiro and Ribak 2006; Thind et al. 2008). Normally, basal dendrites are transient structures on immature DGCs of rodents that do not become synaptically integrated (Seress and Pokorny 1981). After SE, however, synapses rapidly develop onto HBDs (Shapiro et al. 2007), leading to spine formation (Jessberger et al. 2007b; Walter et al. 2007) and an overall increase in primarily excitatory inputs onto the cell (Thind et al. 2008). These HBDs persist once the cell has reached maturity (Ribak et al. 2000; Walter et al. 2007). Recent work also indicates that DGCs with a prominent HBD are more likely to have a very high spine density on their apical dendrites when compared with other DGCs born at the same time (Murphy et al. 2011). The mechanism for the increased presence of DGCs with HBDs after SE is not entirely understood, although the fact that HBDs are part of a normal developmental stage for DGCs may be a clue. Only DGCs that are in the process of developing at the onset of SE, or those that are born afterward, show increased rates of HBD persistence (Jessberger et al. 2007b; Kron et al. 2010; Walter et al. 2007). Thus, it seems more likely that DGCs developing in this abnormal environment are for some reason unable to retract their HBDs, than DGCs that regrow HBDs after they have been retracted. Importantly, HBDs have been linked not only to anatomical measures of excitability but to increased physiological excitation as well (Austin and Buckmaster 2004).

Despite the fact that HEGCs and those with HBDs comprise a minority of the total population, the degree to which they are hyper-innervated suggests that they may have a powerful influence on overall network excitability. Two studies suggest that pro-excitatory changes in a relatively small subset of DGCs are sufficient to induce epileptic activity. In one study, computational modeling of an epileptic dentate gyrus showed that the configuration of synaptic connectivity that most reliably produced seizure-like activity was one in which a small subset of DGCs (5 %) were highly interconnected (Morgan and Soltesz 2008). By keeping constant the total number of synapses in the network, and changing only the distribution of the recurrent DGC inputs, Morgan and Soltesz found that a network containing the highly interconnected DGC "hubs" was strongly activated by a relatively mild input. In a separate study, Pun and colleagues used a conditional transgenic mouse to alter the development of a subset of adult-born DGCs in the context of an otherwise normal brain. The genetically altered DGCs displayed HBDs, increased spine density, and ectopic migration into the hilus, similar to DGCs present in models of epilepsy. Remarkably, although only 9–24 % of DGCs developed these abnormal features, animals subsequently developed spontaneous recurrent seizures (Pun et al. 2012). Together, these studies indicate that small populations of DGCs can play a pivotal role in the development of seizure activity, and they highlight the need for a better understanding of individual DGC abnormalities in the context of TLE.

5.3.4 Axon Reorganization in Epilepsy

Mossy fiber sprouting (MFS) is another important feature of the recurrent epileptic network, but it has been difficult to determine whether specific subpopulations of DGCs selectively participate in this abnormality. Initially, the hypothesis was put forward that adult-generated DGCs developing after an epileptogenic insult are responsible for MFS (Parent and Lowenstein 1997); however, a study using irradiation to suppress neurogenesis provided evidence that ablating DGCs born after SE failed to prevent MFS within 4 weeks after SE (Parent et al. 1999). With the use of the more precise retrovirus birthdating methods, subsequent work suggested that only cells that were developing during SE or born afterward contributed to MFS (Kron et al. 2010). Recently, with an even more refined retrovirus, which targets yellow fluorescent protein to axon terminals by conjugating it to the synaptic vesicle protein synaptophysin (Umemori et al. 2004), we have found that neonatally generated DGCs that are mature at the time of SE do participate in MFS, along with those born during adulthood (unpublished data). Nevertheless, the role of MFS, at least in the supragranular inner molecular layer, in epileptogenesis remains controversial.

5.3.5 Potential Compensatory Role for New DGCs in Epilepsy

As mentioned earlier, some DGCs generated after SE appear to show decreased excitability, perhaps as a means of compensating for the overall hyperexcitability within the network. Several recent studies have used fluorescent reporter labeling using retroviruses or transgenic mice to identify DGCs born after SE in order to characterize the morphological and physiological characteristics of this population. On one extreme, nearly all of the adult-born DGCs examined in an adult rat electrical stimulation-induced SE model displayed strongly reduced excitation and increased inhibition (Jakubs et al. 2006), suggesting an anti-epileptogenic role for this population as a whole. In other studies of rodent chemoconvulsant TLE models, many of the adult-born DGCs display pro-excitatory features and receive increased excitatory inputs (Kron et al. 2010; Walter et al. 2007; Wood et al. 2011). Still others report a more mixed population, in which some cells clearly display pro-excitatory features (Jessberger et al. 2007b), while others have features that are consistent with reduced excitation (Murphy et al. 2011). The variability in the proportion of cells that might be “pro-excitatory” as opposed to “pro-inhibitory” in these models may reflect the use of different SE induction protocols. The type of induction protocol can have a dramatic effect on the development of chronic epilepsy, influencing the number and severity of spontaneous seizures. Moreover, post-SE-generated DGCs that are continuously exposed to seizures during their development show increased excitatory activity, even without a dramatic increase in aberrant morphology (Wood et al. 2011).

5.4 Functional Significance

The structural and functional heterogeneity of DGCs born after SE is one major challenge when considering the best way to target these cells for therapeutic intervention. Certainly aberrant integration of and increased excitatory inputs onto some of these cells seems to indicate that they play a pathological role in the development of spontaneous seizures. Supporting this idea is the fact that as little as 9 % of aberrantly connected DGCs in an otherwise normal animal is sufficient to induce spontaneous seizures (Pun et al. 2012). However, the subset of cells in this same population that display pro-inhibitory features in some epilepsy models may be an important part of the brain's attempt to balance excess network excitability.

5.4.1 Neurogenesis Ablation Studies

Experimental efforts to eliminate the entire population of DGCs that are born in response to SE as a means of understanding the overall impact of this population on seizure development yield mixed results. For example, treatment with an antimitotic agent after chemoconvulsant-induced SE results in reduced seizure frequency (Jung et al. 2004; Jung et al. 2006), suggesting a net excitatory effect of this population on the network. However, focal brain irradiation to suppress neurogenesis in a kindling model increased seizure activity (Raedt et al. 2007), a finding that may indicate a net inhibitory effect of post-SE neurogenesis. Despite the differences in SE models and means of reducing neurogenesis in these experiments, taken together the results suggest that a targeted approach that can address aberrant integration of this cell population without interfering with the development of compensatory mechanisms may be the most effective strategy.

5.4.1.1 Neurogenesis and Human Temporal Lobe Epilepsy

Another, perhaps larger, challenge is to understand how the findings in rodent models relate to the human disease. When considering this question, it is important to note that TLE manifestations in patients are even more diverse than in animal models. Thus, it is perhaps incorrect to try to identify a model that "most closely" resembles the human disease. Instead, it seems most relevant to focus on the salient features in the different models.

Increased neurogenesis, per se, has not been demonstrated in human tissue from adult TLE patients (Fahrner et al. 2007), but there is evidence for increased numbers of neural progenitors in the dentate gyrus of some patients (Crespel et al. 2005). However, one must be cautious when drawing conclusions from postmortem human tissue or specimens obtained from epilepsy surgery to treat drug-resistant seizures.

Often, this tissue comes from patients who have had seizures for many years and therefore may not reflect the same structural changes that initially led to epilepsy development. Because experimental tissue from patients in early stages of TLE is largely unavailable, it has been difficult to determine whether hippocampal neurogenesis is affected early in the disease. Interestingly, there is some evidence of increased neurogenesis in very young children after an epileptogenic insult (Blumcke et al. 2001).

The presence of HEGCs in human TLE tissue is well documented, but is not found in every patient (Parent et al. 2006; Parent and Murphy 2008; Scharfman and Gray 2007). Although lack of HEGCs in some tissues could be due to a number of factors, the same issues related to early versus late stage disease course apply when comparing findings in human tissue to experimental models. In addition to this caveat, another potential difficulty is the fact that the method for identifying cell types based on expression of endogenous markers identified in rodent cells may not be completely effective in human tissue. Thus, the presence of HEGCs could be missed if investigators only use one method for detection (Scharfman and Gray 2007). Nonetheless, HEGCs are present and functionally integrated in tissue from at least some patients with TLE (Parent et al. 2006; Parent and Murphy 2008). No obvious differences in intrinsic measures of excitability or perforant path connectivity are apparent between HEGCs and those in the GCL in human surgical TLE tissue (Parent and Murphy 2008; A. Althaus, G. Murphy and J. Parent, unpublished data). However, this finding is also largely true in animal models (Scharfman et al. 2003) and does not necessarily mean HEGCs do not play important roles in the network-level changes in excitability. Other electrophysiological studies of HEGCs in animal models have not yet been replicated in human tissue. Reasons include the reduced availability of living tissue and the difficulty in controlling for factors like slice angle and location within the rostro-caudal axis within the hippocampus when making acute slices from human tissue.

Although the increased presence of HBDs on developing and newborn DGCs after SE is a feature of many different models and represents an important opportunity for increased recurrent input between DGCs, the relevance of this finding to human TLE is also unclear. While mature DGCs in rodent tissue rarely (<6 %) have HBDs (Kron et al. 2010; Walter et al. 2007), they are more common on DGCs in non-epileptic humans (10 %) and nonhuman primates (25 %) (Seress 1992). However, several studies suggest that the number of cells with HBDs is increased in human TLE (Franck et al. 1995; von Campe et al. 1997), raising the possibility that HBDs contribute to increased recurrent innervation. It is unknown whether inputs to these structures are altered in TLE patients, and more work is needed to understand the potential importance of HBDs in human TLE.

In addition to contributing to seizure generation or spread, the aberrant integration of post-SE born DGCs may have other adverse effects on the epileptic brain. Great interest exists in understanding the normal function of adult neurogenesis, and though there are no definitive answers yet, a number of studies have found links between disrupted neurogenesis and disruptions in learning and memory (Deng et al. 2010). Although seizures in human TLE and in animal models

affect many other structures besides the dentate gyrus, the potential relationship between aberrant neurogenesis and cognitive impairments in epileptic animals and human patients is intriguing. Also of interest is the reduction of neurogenesis that occurs in some animals in later stages of chronic TLE (Hattiangady et al. 2004) and the increased incidence of major depression in patients with TLE (Hermann et al. 2000). Because the presence of DGC neurogenesis appears to be a critical aspect of the effect of antidepressants, at least in some mouse strains (Santarelli et al. 2003), patients with TLE and comorbid major depression may benefit doubly from a therapeutic target that corrects aberrant neurogenesis or stimulates normal neurogenesis.

5.5 Conclusions

Seizure activity has such profound and widespread effects on all aspects of neurogenesis in the dentate gyrus that it may not be possible to understand the implications of any single aberrant feature on its own. Similarly, nearly all TLE models disrupt the normal function of many brain regions, making it extremely difficult to determine the "net" contribution of aberrant neurogenesis to epilepsy-related pathophysiology in animal models as well as human disease. However, the robust and reproducible nature of the data implicating aberrant DGC neurogenesis in the pathogenesis of TLE indicates the need for a more comprehensive understanding of how aberrant features develop and highlights the exciting potential for targeting aberrant neurogenesis as a therapeutic strategy. An important consideration going forward is the heterogeneity of DGC responses to seizures. In particular, much more work is needed to determine the potential compensatory or "anti-epileptogenic" function of some subpopulations of DGCs. A better understanding of the factors that influence DGCs to develop either pro- or anti-excitatory features should be useful in developing treatments not only for TLE but also for other neurological conditions that are believed to involve dysregulated neurogenesis.

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Chapter 6
Neurogenesis and Gliogenesis in the Postnatal Hypothalamus: A New Level of Plasticity for the Regulation of Hypothalamic Function?

Ariane Sharif, Sergio R. Ojeda, and Vincent Prevot

Abbreviations

AgRP	Agouti-related peptide
APC	Adenomatosis polyposis coli
Ara-C	Cytosine-β-D-arabinofuranoside
ARC	Arcuate nucleus of the hypothalamus
AVPV	Anteroventral periventricular nucleus of the hypothalamus
BDNF	Brain-derived neurotrophic factor
BLBP	Brain lipid-binding protein
BrdU	5-bromo-2′-deoxyuridine
CNPase	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNTF	Ciliary neurotrophic factor
CVOs	Circumventricular organs
Dcx	Doublecortin
DMH	Dorsomedial hypothalamus
E	Embryonic day
EdU	5-Ethynyl-2′-deoxyuridine
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FGF2	Fibroblast growth factor 2

A. Sharif (✉) • V. Prevot (✉)
French Institute of Health and Medical Research, Jean-Pierre Aubert Research Center, Development and Plasticity of the Postnatal Brain, Unit 837, University of Lille 2, School of Medicine, Lille, France
e-mail: ariane.sharif@inserm.fr; vincent.prevot@inserm.fr

S.R. Ojeda
Division of Neuroscience, Oregon National Primate Research Center-Oregon Health & Science University, Beaverton, OR 97006, USA

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3_6, © Springer Science+Business Media New York 2014

FGF10	Fibroblast growth factor 10
GalC	Galactocerebrosidase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLAST	Glutamate/aspartate transporter
GnRH	Gonadotropin-releasing hormone
Gpr50	G-protein-coupled receptor 50
HFD	High-fat diet
i.c.v.	Intracerebroventricular
IGF-I	Insulin-like growth factor I
IKKβ	IκB kinase β
IL-1β	Interleukin 1β
LHA	Lateral hypothalamic area
Lhx2	LIM homeobox 2
MAP2	Microtubule-associated protein 2
ME	Median eminence
MSG	Monosodium glutamate
NeuN	Neuron-specific nuclear protein
NF-κB	Nuclear factor-κB
NPY	Neuropeptide Y
OVLT	Organum vasculosum of the lamina terminalis
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PLP	Myelin proteolipid protein
POMC	Pro-opiomelanocortin
Rax	Retina and anterior neural fold homeobox
SDN	Sexually dimorphic nucleus of the preoptic area
SGZ	Subgranular zone
STAT3	Signal transducer and activator of transcription 3
SVZ	Subventricular zone
TNFα	Tumor necrosis factor α
TrkB	The high-affinity receptor for BDNF
TUNEL	Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling
VMH	Ventromedial hypothalamus
YFP	Yellow fluorescent protein

6.1 Introduction

The concept of plasticity in the mature mammalian central nervous system was profoundly challenged by the discovery that neurogenesis persists during postnatal life. Two neurogenic regions have been described in the mature brain, the subventricular zone (SVZ) of the lateral ventricles, which gives birth to neurons that

migrate long distances to integrate into the olfactory bulbs, and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus, which adds new neurons locally to the granule cell layer of the hippocampus. Nevertheless, since the first observations in the 1960s that cell proliferation occurred in the adult brain, it took decades before these two neurogenic niches were actually recognized as functional and meaningful sources of cellular plasticity in the mature brain. Since then, intense research in this field has yielded an ever-increasing fount of knowledge regarding the cellular and molecular determinants of adult neurogenesis in these regions, such as the structure of the niches and the identity, lineage, and molecular control mechanisms of the constituent stem cells. Even though the functions of this process are far from completely elucidated, a number of studies have implicated adult neurogenesis in learning and memory (for reviews, see Gross 2000; Alvarez-Buylla et al. 2001; Alvarez-Buylla and Lim 2004; Ming and Song 2005; Lledo et al. 2006). Besides these two primary neurogenic niches, the production of new neurons has been reported in other brain locations such as the neocortex, the piriform cortex, the olfactory bulbs, the striatum, the amygdala, the substantia nigra, and the dorsal vagal complex, although whether neurogenesis in these regions occurs under physiological conditions is still a matter of debate (Migaud et al. 2010; Emsley et al. 2005; Gould 2007). The situation appears to be different in the hypothalamus, a small ventromedial brain structure controlling key physiological functions such as energy homeostasis, reproductive function, thermoregulation, and circadian rhythms. Indeed, since the first report of cell proliferation in the hypothalamus of adult hamsters (Huang et al. 1998), a growing number of studies have documented both neurogenesis and gliogenesis in the postnatal hypothalamus of various mammalian species. Here, we (1) review current evidence to support the existence of neurogenesis and gliogenesis in the postnatal mammalian hypothalamus, (2) discuss the proposed models of hypothalamic stem cell lineage, (3) present current knowledge regarding the intrinsic and extrinsic factors that modulate the generation of new hypothalamic neural cells, and (4) provide new data indicating the possible involvement of this process in the pathophysiology of a major hypothalamus-dependent function: the control of energy metabolism.

6.2 Evidence for Ongoing Neurogenesis and Gliogenesis in the Postnatal Mammalian Hypothalamus

6.2.1 The Hypothalamus Exhibits Constitutive Endogenous Cell Proliferation

Several studies have now reported the presence in vivo of proliferative cells in the postnatal hypothalamus of the rat (Pencea et al. 2001; Xu et al. 2005a; Perez-Martin et al. 2010; Ahmed et al. 2008), mouse (Kokoeva et al. 2007; McNay et al. 2012; Pierce and Xu 2010; Li et al. 2012; Haan et al. 2013; Lee et al. 2012; Bennett et al. 2009;

Hourai and Miyata 2013; Morita et al. 2013; Robins et al. 2013), prairie and meadow vole (Fowler et al. 2002, 2005), hamster (Huang et al. 1998), sheep (Migaud et al. 2010, 2011), and pig (Rankin et al. 2003). In most of these studies, dividing cells were identified using the thymidine analog 5-bromo-2′-deoxyuridine (BrdU), which is incorporated into DNA during the S-phase of the cell cycle and is a widely accepted marker of cell division (Emsley et al. 2005; Cooper-Kuhn and Kuhn 2002; Bauer and Patterson 2005). In support of their proliferative nature, BrdU-labeled cells often appear as pairs of closely apposed cells (Migaud et al. 2010, 2011; Pencea et al. 2001; Xu et al. 2005a; Perez-Martin et al. 2010; Ahmed et al. 2008; Kokoeva et al. 2005, 2007; Pierce and Xu 2010; Haan et al. 2013) (Fig. 6.1a), suggestive of recently divided daughter cells. The Ki-67 protein, an endogenous cell proliferation marker that is present during all active phases of the cell cycle (Scholzen and Gerdes 2000), is detected in subsets of BrdU-labeled hypothalamic cells (Kokoeva et al. 2007; Lee et al. 2012; Migaud et al. 2011). The presence of dividing cells in the postnatal hypothalamus is further suggested by the detection of cells labeled with another nucleotide analog, tritiated thymidine (Perez-Martin et al. 2010), and cells expressing proliferating cell nuclear antigen (PCNA), another endogenous cell-cycle marker (McNay et al. 2012; Pierce and Xu 2010). It should be mentioned that incorporation of BrdU and expression of endogenous cell-cycle markers can also occur in postmitotic cells undergoing DNA repair or apoptosis (Kuan et al. 2004; Rakic 2002). Nevertheless, the absence of terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining, a marker of apoptosis, in BrdU-labeled cells in the postnatal hypothalamus strongly supports the proliferative nature of these cells (McNay et al. 2012).

Most of the abovementioned studies report a low—yet variable—rate of constitutive proliferation in the hypothalamus, which has likely contributed to its delayed recognition. Indeed, low-sensitivity protocols hardly detect proliferative cells in the hypothalamus, even though they enable the visualization of dividing cells in the SVZ adjacent to the lateral ventricle (Pencea et al. 2001), suggesting that DNA synthesis in the hypothalamus occurs at a low rate compared to the SVZ. Moreover, variable proliferation rates across studies can be explained by several factors, such as the dosage, length, and mode of BrdU administration; the species, sex, and genetic background of the animals; and the quantification method. In addition, proliferative activity in the hypothalamus has been shown to decrease sharply with age (Haan et al. 2013; Lee et al. 2012). Some methodological concerns have been addressed in comparative studies showing that the continuous intracerebroventricular (i.c.v.) infusion of BrdU via implanted minipumps labels a much greater number of proliferative cells in the adult rodent hypothalamus than repeated intraperitoneal injections of BrdU for the same duration of time (Pencea et al. 2001; Kokoeva et al. 2007). Haan et al. (2013) compared the effectiveness of BrdU administered via drinking water to that of intraperitoneal injection of its less toxic analog, 5-ethynyl-2′-deoxyuridine (EdU), to detect proliferative activity in the hypothalamus of postnatal mice. They found that, although the distribution profile of labeled cells was similar with both protocols, 70 % fewer proliferating cells were detected in EdU-treated than in BrdU-treated animals. Thus, the recognition and quantification of

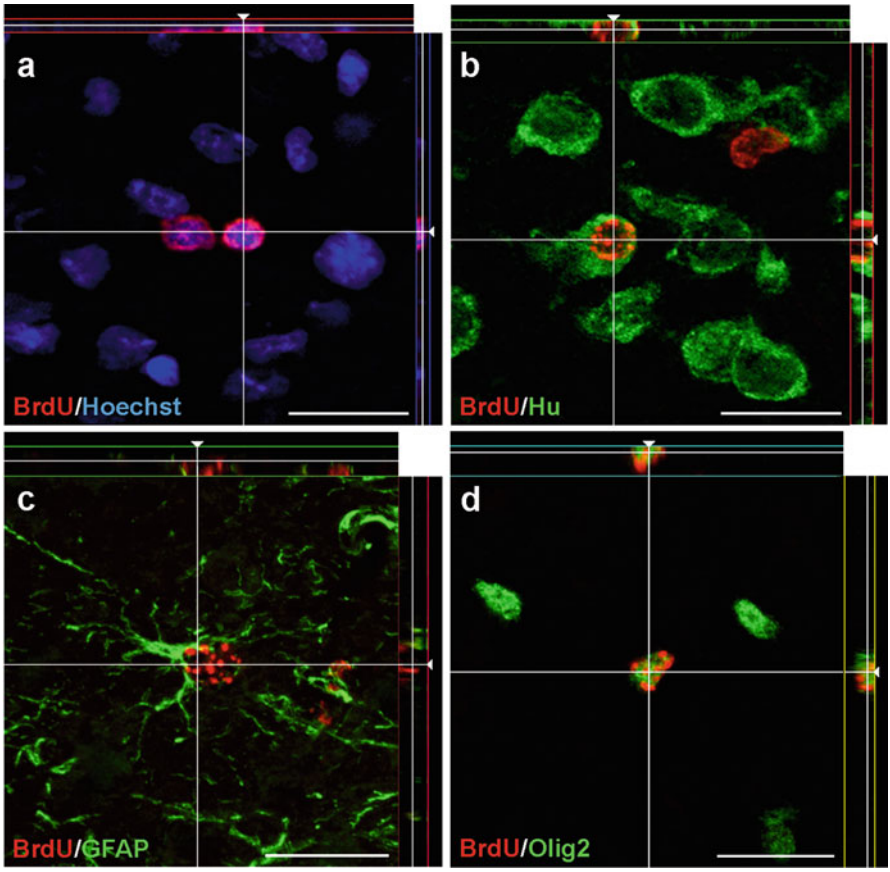

Fig. 6.1 BrdU-based protocol to reveal neurogenesis and gliogenesis in the postnatal hypothalamus. Sprague–Dawley rats received a single intraperitoneal injection of BrdU (300 mg/kg; Sigma) at either 12 days (**a**) or 8 days of age (**b**–**d**) and were allowed to survive 24 h (**a**) or 7 days (**b**–**d**) before euthanasia. Cryostat sections (14 μm) of the hypothalamus were subjected to immunodetection of BrdU (*red*) and markers of the different neural cell lineages (*green*). (**a**) Twenty-four hours after BrdU injection, BrdU-labeled cells often appeared as pairs of closely apposed cells, a picture suggestive of a recent mitotic event. Cell nuclei were counterstained with Hoechst (*blue*). When rats were euthanized 7 days after BrdU injection to allow newborn cells to engage in the differentiation process, subsets of BrdU-labeled newborn cells were found to express the neuronal marker Hu (**b**), the astrocytic marker GFAP (**c**), or the oligodendrocytic marker Olig2 (**d**). Scale bars = 20 μm

cell proliferation in the postnatal hypothalamus, which occurs at a low rate, is highly dependent on the method of detection and the mode of administration (and hence the dose to which cells are exposed). Although the variety of protocols used makes it difficult to draw definitive conclusions as to the absolute rate of cell proliferation in this region, the data reviewed here support the existence of constitutive cell proliferation in the hypothalamus at postnatal ages.

6.2.2 *New Neurons and Glial Cells Are Generated in the Postnatal Hypothalamus In Vivo*

Using neuronal and glial phenotypic markers, proliferative hypothalamic cells have been shown to give rise to mature neural cells. Two main in vivo tracking approaches have been employed to identify the fate of these newborn cells. Most studies rely on the co-detection of BrdU, which is transmitted to the progeny of the original incorporating cell, and phenotypic markers of neurons and glia (Fig. 6.1). More recently, transgenic mouse lines expressing a reporter gene under the control of a specific promoter have been used to fate-map the descendants of distinct cell populations. The phenotypic identity of tracked cells is determined by evaluating the co-expression of the reporter protein with phenotypic markers of mature neural cells (Li et al. 2012; Haan et al. 2013; Lee et al. 2012; Robins et al. 2013).

The neuronal nature of newborn cells has been assessed using several pan-neuronal lineage markers, such as Hu and βIII-tubulin (TuJ1), two markers of immature and mature neurons (Lee et al. 1990; Marusich et al. 1994); doublecortin (Dcx), a microtubule-associated protein transiently expressed in newly generated migratory neuroblasts (Brown et al. 2003; Rao and Shetty 2004; Couillard-Despres et al. 2005); and neuron-specific nuclear protein (NeuN) and microtubule-associated protein 2 (MAP2), two markers of mature neurons (Mullen et al. 1992; Bernhardt and Matus 1984). Ultrastructural features have also been evaluated, such as the presence of synapses. These experimental approaches and tools have enabled several groups to demonstrate that cells exhibiting a neuronal phenotype are born in the early postnatal and adult rodent (Huang et al. 1998; Pencea et al. 2001; Xu et al. 2005a; Perez-Martin et al. 2010; Kokoeva et al. 2007; Li et al. 2012; Haan et al. 2013; Lee et al. 2012; Robins et al. 2013; Fowler et al. 2002) and sheep (Migaud et al. 2011) hypothalamus. The expression of specific phenotypic markers in newly generated hypothalamic neurons has also been documented. In particular, newborn neurons that express neuropeptide Y (NPY) and pro-opiomelanocortin (POMC), two neuropeptides critically involved in the control of food intake and body weight (Morton et al. 2006; Myers and Olson 2012; Williams and Elmquist 2012), have been detected in the early postnatal (Lee et al. 2012) and adult (Li et al. 2012) mouse hypothalamus. Newly generated vasopressin-containing neurons have been observed in the pig hypothalamus (Rankin et al. 2003). Moreover, upon treatment with fibroblast growth factor 2 (FGF2), which amplifies a pool of presumptive neural progenitor cells in the ependymal layer of the third ventricle of adult rats, newborn neurons expressing orexin A, a hypothalamus-specific neuropeptide that regulates feeding behavior (Sakurai et al. 1998), have been observed in the lateral hypothalamus (Xu et al. 2005a).

Even though neurogenesis is consistently detected in the hypothalamic parenchyma, its rate, i.e., the fraction of newborn cells that acquire a neuronal fate, is highly variable among studies. In the adult rat hypothalamus, the estimated rate of neurogenesis varies from less than 1 to 37 %, depending on the study (Pencea et al. 2001; Xu et al. 2005a; Perez-Martin et al. 2010). Such variability likely reflects the

great disparity in experimental conditions and settings, in particular the modalities of BrdU treatment (see above), the time of analysis after BrdU administration, and the neuronal markers used for quantification. A BrdU-labeling study of the arcuate nucleus (ARC) of adult mice reported a rate of neurogenesis of more than 80 % (Li et al. 2012). Genetic fate-mapping studies of hypothalamic neural stem/progenitor cells in adult mice have revealed highly variable rates of neuronal fate commitment depending on the promoter used and hence on the population of tracked cells. Indeed, fibroblast growth factor 10-expressing ($FGF10^+$) neural stem/progenitor cells differentiate at more than 80 % into neurons (Haan et al. 2013), while glutamate/aspartate transporter-expressing ($GLAST^+$) neural stem/progenitor cells generate less than 2 % neurons (Robins et al. 2013). Another important issue is the extent of hypothalamic neurogenesis. Indeed, a high rate of neuronal fate commitment in the face of a low rate of cell birth may not contribute significantly to the remodeling of neuronal circuits. A lineage-tracing study of nestin-expressing progenitors in mice showed that 160 new neurons were generated in the median eminence between postnatal day (P) 4 and P35, accounting for 8 % of the entire neuronal population of this region (Lee et al. 2012). Notably, given that the adult median eminence contains few if any neurons, we speculate that these early-born neurons migrate to neighboring parenchymal regions such as the ARC or do not survive to adulthood. Similarly, by injecting a lentiviral reporter construct into the mediobasal hypothalamus of adult mice, Li and colleagues fate-mapped Sox2-expressing progenitors and reported the generation of ~1,000 new neurons in the ARC over an 80-day period, estimated to account for 6 % of the neuronal population of this region (Li et al. 2012). Among these newborn neurons, 10 % were POMC neurons and 3 % were NPY neurons, indicating a slow rate of neuronal replacement within these specific cell populations. Interestingly, McNay and colleagues used another strategy to evaluate the postnatal turnover of neurons in the mouse ARC. Given that POMC and NPY neurons are born between embryonic day (E) 10.5 and E12.5 (McNay et al. 2006; Pelling et al. 2011; Padilla et al. 2010), they injected BrdU at E10.5 to label ARC neurons and then assayed the replacement of these embryo-born BrdU-labeled neurons by new unlabeled neurons at several postnatal ages to obtain an estimate of their turnover. The results showed that more than half of the embryo-born BrdU-labeled POMC and NPY neurons present at 4 weeks of age disappeared during the following 8 weeks, while the integrity of the ARC was maintained, suggesting that these neuronal populations had undergone substantial turnover during this postnatal period (McNay et al. 2012). Altogether, these results suggest that hypothalamic neurogenesis occurs at a relatively low rate compared to the primary neurogenic niches of the SVZ and SGZ, which give birth to thousands of new neurons each day (Peterson 2002; Cameron and McKay 2001). However, it should be noted that the tracking methods used in the two studies mentioned above are unlikely to label the entire pool of neuronal progenitors, leading to a potential underestimation of the true extent of hypothalamic neurogenesis. Moreover, some key hypothalamic functions are regulated by a small number of neurons, as is the case for POMC neurons, which consist of a population of ~3,000 cells in the adult murine hypothalamus (McNay et al. 2006, 2012). Therefore, even if generated at

low rates, newborn neurons may potentially have a significant impact on the reshaping and activity of specific hypothalamic neuronal circuits.

The generation of new glial cells has also been reported under physiological conditions in the adult rodent hypothalamus. A rate of astrogliogenesis of 3 % has been reported in the adult rat hypothalamus, as assessed by the expression of the astrocytic marker glial fibrillary acidic protein (GFAP) (Pencea et al. 2001), and a rate of 6 % has been reported in the mediobasal hypothalamus of adult mice, as evaluated by the expression of S100β, indicating that ~80 astrocytes are generated in this region over an 80-day period (Li et al. 2012). Long-term lineage tracing of a population of GLAST^{+} neural stem/progenitor cells in adult mice has revealed that 44 % of their descendants express GFAP, corresponding to the generation of ~20 new astrocytes in the hypothalamic parenchyma after 9 months (Robins et al. 2013). Other authors have detected few if any newborn GFAP-expressing astrocytes in the hypothalamic parenchyma of adult mice (Kokoeva et al. 2007). It should be noted, however, that the true rate of astrogliogenesis in the hypothalamus might be underestimated, because of the apparent lower density of GFAP-expressing astrocytes in the hypothalamus compared to other structures such as the hippocampus (Kokoeva et al. (2007) and our own observations) and the difficulty in unambiguously identifying astrocytes using cytoskeletal markers. A few newborn oligodendrocytes have been detected in the adult mouse hypothalamus using antibodies to CNPase (RIP) and APC (adenomatosis polyposis coli) (Kokoeva et al. 2007; Li et al. 2012), but the rate of oligodendrogenesis is less than 1 %, corresponding to the birth of eight oligodendrocytes over an 80-day period (Li et al. 2012). Using galactocerebrosidase (GalC) and the myelin proteolipid protein (PLP) as markers, another study has also reported negligible oligodendrogenesis in the adult rat hypothalamus (Pencea et al. 2001). From the studies described above, which have focused on cell specification in the mediobasal hypothalamus, particularly in nuclei related to energy-balance regulation such as the ARC, most hypothalamic neural stem/progenitor cells appear to be highly neurogenic but poorly gliogenic. However, astrogliogenesis is high in the organum vasculosum of the lamina terminalis (OVLT) and in the median eminence of adult mice, two circumventricular organs (CVOs) that like other CVOs in the brain lack a fully functional blood–brain barrier (Langlet et al. 2013a). S100β expression is detected in 30–50 % of newborn cells in the median eminence and in 45 % of newborn cells in the OVLT (Bennett et al. 2009; Morita et al. 2013). In contrast, low neurogenic activity is reported in these regions, with neurogenesis rates ranging from 0 to 18 % depending on the study (Haan et al. 2013; Lee et al. 2012; Bennett et al. 2009; Hourai and Miyata 2013). Similarly, the phenotype of newborn cells during puberty is different in two sexually dimorphic hypothalamic regions, the anteroventral periventricular nucleus of the hypothalamus (AVPV) and the sexually dimorphic nucleus of the preoptic area (SDN). While most newborn cells in the AVPV express NeuN and none express GFAP, newborn cells in the SDN express none of these markers and thus may represent a population of cells that remain undifferentiated (Ahmed et al. 2008). Thus, it seems that the fate specification of newborn cells is differentially regulated between distinct hypothalamic regions in the postnatal brain. Moreover, while neurogenesis in the adult

mouse brain mainly occurs in the parenchymal nuclei latero-dorsal to the median eminence, such as the ARC, neurons born during early postnatal development are mostly found in the median eminence (Lee et al. 2012), suggesting that there might be a temporal switch in the phenotypic fate of newborn cells in these hypothalamic regions during the postnatal period.

6.2.3 Neural Stem/Progenitor Cells Can Be Isolated from the Postnatal Hypothalamus

The presence of neural stem/progenitors in the postnatal hypothalamus has also been assessed using in vitro approaches. The chief method of identifying neural stem cells is the neurosphere assay (Reynolds and Weiss 1992, 1996), in which the tissue of interest is dissociated and grown in a defined medium devoid of serum but supplemented with a cocktail of growth factors, usually FGF2 and epidermal growth factor (EGF). Under these conditions, the presence of neural stem cells is documented by three criteria: (a) the formation of floating spheres of proliferating cells (the so-called neurospheres), (b) the formation of secondary neurospheres from single primary neurosphere cells, and (c) the appearance of cells that express markers for the three neural cell types—neurons, astrocytes, and oligodendrocytes—under the appropriate differentiation conditions (usually an adherent substrate and media supplemented with serum and/or specific factors). Following these criteria, cells forming neurospheres that can be expanded and passaged over time (Xu et al. 2005a; McNay et al. 2012; Li et al. 2012; Robins et al. 2013; Weiss et al. 1996; Haan and Hajihosseini 2009), exhibiting self-renewal properties after clonal analysis (Xu et al. 2005a; Weiss et al. 1996), and giving rise to cells expressing markers for the three neural lineages (Xu et al. 2005a; Li et al. 2012; Robins et al. 2013; Weiss et al. 1996; Haan and Hajihosseini 2009) have been isolated from the adult rodent hypothalamus. Notably, while the hypothalamus yields fewer primary neurospheres than the region containing the SVZ (Weiss et al. 1996), it generates a number of neurospheres comparable to the hippocampal dentate gyrus containing the SGZ (Li et al. 2012). In addition, other authors have used adherent culture conditions to isolate neuronal progenitors from the hypothalamus of young postnatal (Markakis et al. 2004) and adult (Evans et al. 2002) rats. Thus, the adult rodent hypothalamus contains cells that exhibit in vitro the canonical properties of neural stem cells.

6.3 Identity and Location of Hypothalamic Stem Cells

Even though neural stem/progenitor cells have now been consistently reported to be present within the hypothalamus, controversy remains as to their precise identity and location. Two main and nonexclusive hypotheses are being proposed, one

attributing neurogenesis and gliogenesis to a population of neural stem/progenitor cells that reside within the hypothalamic parenchyma and the other designating tanycytes, specialized ependymoglial cells that line the third ventricle wall, as the hypothalamic stem cells (Fig. 6.2).

6.3.1 Parenchymal Location of Hypothalamic Neural Stem Cells?

Several studies have shown that cells divide in situ within the hypothalamic parenchyma. Indeed, closely apposed pairs of BrdU-labeled cells, reminiscent of a recent mitotic event, are seen in the hypothalamic parenchyma of adult rodents (Pencea et al. 2001; Perez-Martin et al. 2010; Kokoeva et al. 2007; Haan et al. 2013) and sheep (Migaud et al. 2011), sometimes at a considerable distance from the third ventricle. Moreover, some of these parenchymal BrdU-labeled cells express the endogenous proliferation marker Ki-67, indicating that they are in an active phase of the cell cycle (Kokoeva et al. 2007). In addition, Sox2, a marker of neural stem/progenitor cells (Suh et al. 2007), is expressed by proliferative cells within the hypothalamic parenchyma of adult rats (Bennett et al. 2009) and sheep (Migaud et al. 2011) and is significantly colocalized with nestin, another marker of neural stem/progenitor cells (Gilyarov 2008) in the mediobasal hypothalamus of adult mice (Li et al. 2012). Lineage tracing of Sox2-expressing cells in adult mice shows that these cells expand and are multipotent in situ (Li et al. 2012). Nevertheless, proliferation and multipotency are not sufficient to determine "stemness": self-renewal and the long-term maintenance of all these properties are additional requirements. It should be mentioned that all in vitro studies that have successfully isolated hypothalamic stemlike cells (see Sect. 6.2.3) have used dissected hypothalamic tissue that included the third ventricle wall. In studies in which cultures were prepared from the hypothalamic parenchyma distant from the third ventricle, no spheres could be obtained (Robins et al. 2013; Weiss et al. 1996), suggesting that stem cells reside in or close to the ventricular wall. Whether the hypothalamic parenchyma also contains neural stem cells, only harbors more restricted multipotent neural progenitors, or contains both cell populations is currently unknown.

6.3.2 Tanycytes: "True" Hypothalamic Neural Stem Cells?

Tanycytes are a population of specialized unciliated ependymoglial cells that line the floor of the third ventricle. They are characterized by a radial morphology, with cell bodies located in the wall of the third ventricle and elongated processes that extend into the parenchyma, and are thought to be persistent radial glial cells in the mature brain (Rodriguez et al. 2005). Tanycytes are involved in the regulation of key

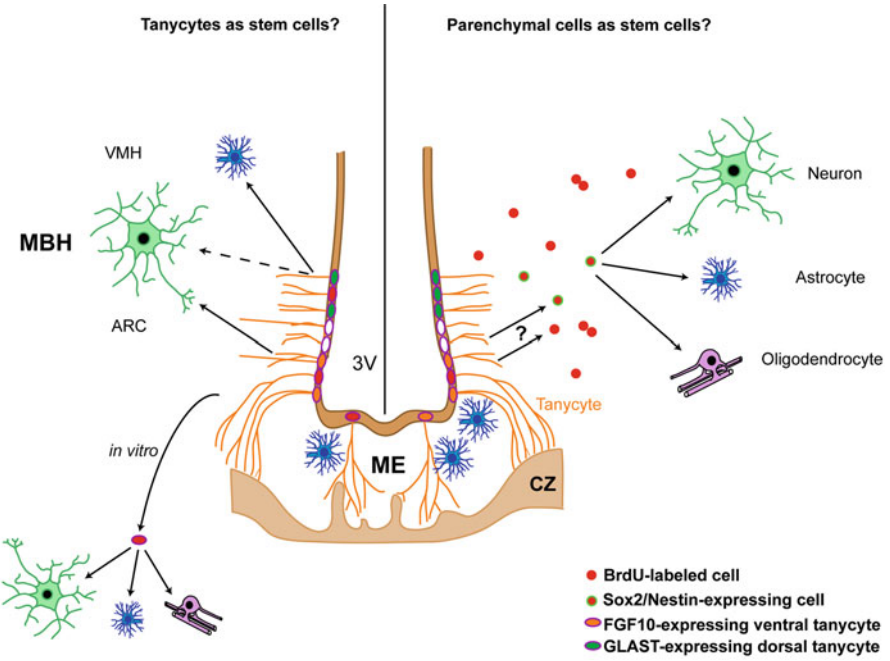

Fig. 6.2 The two principal hypotheses concerning the identity and location of adult hypothalamic stem cells. (*Left*) Tanycytes are the hypothalamic stem cells. Tanycytes express neural stem/progenitor cell markers (Perez-Martin et al. 2010; Li et al. 2012; Haan et al. 2013; Lee et al. 2012; Bennett et al. 2009; Robins et al. 2013; Rodriguez et al. 2005; Mullier et al. 2010; Langlet et al. 2013b; Wei et al. 2002; Baroncini et al. 2007; Berger and Hediger 2001; Sharif et al. 2013; Miranda-Angulo et al. 2013; Batailler et al. 2012; Sidibe et al. 2010), exhibit low proliferative activity, and are neurogenic and gliogenic in situ (Xu et al. 2005a; Haan et al. 2013; Lee et al. 2012; Robins et al. 2013; Rodriguez et al. 2005). Note that tanycytes are heterogeneous with regard to their molecular and functional properties. While ventral FGF10-expressing tanycytes are neurogenic (Haan et al. 2013), dorsal GLAST-expressing tanycytes are highly gliogenic but poorly neurogenic (Robins et al. 2013). Also note that while adult ventral tanycytes give rise to neurons that populate the mediobasal hypothalamus (Haan et al. 2013), ventral tanycytes give rise to neurons that are detected within the median eminence during early postnatal development (not shown) (Lee et al. 2012). Given that the adult median eminence contains few if any neurons, these early-born median eminence neurons may subsequently die or migrate to neighboring hypothalamic regions. Moreover, in vitro studies suggest that tanycytes exhibit canonical neural stem cell properties (Xu et al. 2005a; Bennett et al. 2009; Robins et al. 2013; Allet et al. 2006). (*Right*) Stem cells reside within the mediobasal hypothalamic parenchyma. Cells that are able to divide, sometimes at a long distance from the ventricle (BrdU-labeled *red* cells) (Pencea et al. 2001; Perez-Martin et al. 2010; Kokoeva et al. 2007; Haan et al. 2013; Migaud et al. 2011), that express the neural stem/progenitor cell markers nestin and Sox2 (Li et al. 2012; Bennett et al. 2009; Migaud et al. 2011), and that give rise to the three neural cell lineages in situ (neuronal, astroglial, and oligodendroglial) (Li et al. 2012) are found in the mediobasal hypothalamus. It should be mentioned that the higher proliferative activity of parenchymal cells compared to tanycytes (Pencea et al. 2001; Kokoeva et al. 2007; Haan et al. 2013), associated with the inability to isolate stemlike cells when tissue dissections do not include the ventricular region (Robins et al. 2013; Weiss et al. 1996), argue in favor of tanycytes being the true stem cells and dividing parenchymal cells being multipotent progenitors. It also remains to be determined whether a lineage relationship exists between these putative tanycytic stem cells and putative parenchymal stem/progenitor cells (see main text for details). *3V* third ventricle, *ARC* arcuate nucleus of the hypothalamus, *CZ* capillary zone, *MBH* mediobasal hypothalamus, *ME* median eminence, *VMH* ventromedial hypothalamus

hypothalamic functions such as reproduction and metabolism through their ability to dynamically control hypothalamic neuropeptide secretion into the portal vasculature (Prevot et al. 2010), to sense blood glucose levels (Frayling et al. 2011), and to regulate blood–brain exchanges via their barrier properties (Rodriguez et al. 2005; Mullier et al. 2010; Langlet et al. 2013b; Myers 2013). Moreover, accumulating data suggest that tanycytes are endowed with neural stem cell-like properties. First, they share a number of phenotypic markers with neural stem/progenitor cells, such as nestin (Haan et al. 2013; Lee et al. 2012; Bennett et al. 2009; Robins et al. 2013; Wei et al. 2002; Baroncini et al. 2007) (Fig. 6.3a), GFAP (Perez-Martin et al. 2010; Haan et al. 2013; Robins et al. 2013) (Fig. 6.3c, d, f), vimentin (Perez-Martin et al. 2010; Bennett et al. 2009; Robins et al. 2013; Rodriguez et al. 2005; Mullier et al. 2010; Langlet et al. 2013b; Baroncini et al. 2007) (Fig. 6.3b), brain lipid-binding protein (BLBP), Musashi (Haan et al. 2013), Sox2 (Li et al. 2012; Haan et al. 2013; Lee et al. 2012; Robins et al. 2013), GLAST (Haan et al. 2013; Robins et al. 2013; Berger and Hediger 2001; Sharif et al. 2013) (Fig. 6.3e, f), components of the Notch pathway, and several other genes that are selectively expressed by hypothalamic progenitor cells such as *Gpr50*, *Rax*, and *Lhx2* (Lee et al. 2012; Miranda-Angulo et al. 2013; Batailler et al. 2012; Sidibe et al. 2010). It should be noted that expression of GFAP and GLAST is restricted to subpopulations of tanycytes (Haan et al. 2013; Robins et al. 2013; Berger and Hediger 2001) (Fig. 6.3c–f). Second, tanycytes divide in situ in the young postnatal (Haan et al. 2013; Lee et al. 2012; Rodriguez et al. 2005) and adult (Xu et al. 2005a; Haan et al. 2013; Robins et al. 2013) rodent hypothalamus. Importantly, most studies report a very low number of dividing cells in the ependymal layer of the third ventricle, which includes tanycytes, most proliferative cells being seen in the parenchymal compartment of the adult hypothalamus (Pencea et al. 2001; Kokoeva et al. 2007; Haan et al. 2013). In primary neurogenic niches, neural stem cells appear to be relatively quiescent cells that give rise to more restricted and actively dividing progenitors (Alvarez-Buylla and Lim 2004; Morshead et al. 1994). Therefore, the rare occurrence of cell division in tanycytes in situ compared to the higher proliferative activity of cells in the parenchyma argues

Fig. 6.3 (continued) GFAP is also detected in tanycytic processes within the parenchyma (*arrows*). (**e**, **f**) Co-detection of GLAST (*red*) and GFAP (*green*) in adult mouse tanycytes. To visualize GLAST expression, we used tamoxifen-treated GLAST^{creERT2}:ACTB-td^{Tomato} double transgenic mice (Sharif et al. 2013). (**e**) At the level of the median eminence, GLAST expression is detected in some tanycytic cell bodies (*arrowheads*) and in tanycytic processes (*arrows*) that reach the external zone of the median eminence where they contact portal blood capillaries. While GFAP is strongly expressed in astrocytes located underneath the tanycyte-containing ependymal layer, it is not detected in tanycytes. (**f**) Dorsal tanycytes facing the VMH express GLAST and GFAP in non-overlapping populations. Co-expression of the two proteins was seen in only one tanycytic process (*arrow*). Note that GLAST is expressed in cells with astrocytic morphology within the parenchyma (*arrowheads*), some of which co-express GFAP (*crossed arrows*). (**d**, **e**) Cell nuclei were counterstained with Hoechst (*blue*). *3V* third ventricle, *ARC* arcuate nucleus of the hypothalamus, *DMH* dorsomedial hypothalamus, *ME* median eminence, *VMH* ventromedial hypothalamus. Scale bars: 200 μm (**a**, **b**) and 50 μm (**c**, **d**, **e**, **f**)

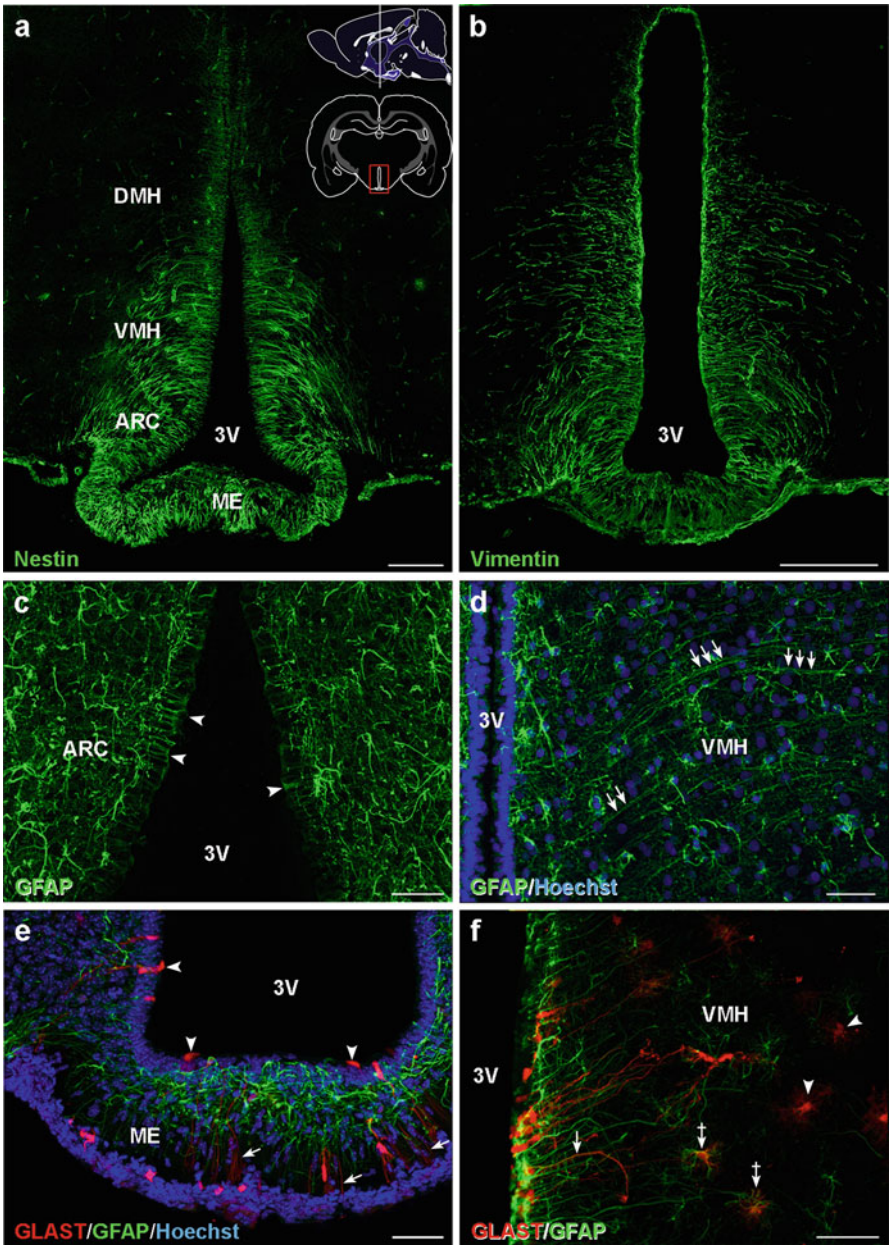

Fig. 6.3 Adult rodent tanycytes express neural stem/progenitor cell markers. Immunodetection of nestin (*green*) in adult rat tanycytes (**a**) and vimentin (*green*) in adult mouse tanycytes (**b**). *Inset* in (**a**) shows a sagittal (*top*) and coronal (*bottom*) view of the hypothalamic region depicted in this figure (drawn from the stereotaxic rat brain atlas of Paxinos and Watson (1982)). (**c**, **d**) Expression of GFAP (*green*) in adult rat tanycytes. *Arrowheads* in **c** point to GFAP-expressing tanycytic cell bodies lining the ventricle at the level of the ARC. (**d**) More dorsally, at the level of the VMH,

in favor of tanycytes being stem cells and dividing parenchymal cells being merely local progenitors.

As a consequence of their relative quiescence, stem cells remain labeled for extended periods of time after thymidine analog incorporation (Potten and Morris 1988; Cotsarelis et al. 1989; Doetsch et al. 1999a), while actively dividing progenitors lose their labeling due to serial dilution over successive divisions. Notably, when we injected rats with a pulse of BrdU during early postnatal life, BrdU-labeled tanycytes, some of which expressed the cell-cycle marker PCNA, were seen in adult animals (C. Allet and V. Prevot, unpublished data). These results suggest that tanycytes, which had incorporated BrdU early in life, had retained their labeling until adulthood due to their relative quiescence, while still remaining capable of entering the cell cycle, as attested to by their expression of PCNA. These proliferative characteristics are strongly suggestive of a stem cell identity. Third, lineage-tracing studies have revealed that tanycytes are neurogenic and gliogenic in vivo. The genetic fate mapping of hypothalamic tanycytes in mice via the inducible nestin-driven expression of a fluorescent protein suggests that tanycytes generate mostly neurons in the median eminence during early postnatal life (Lee et al. 2012). In adult mice, genetic fate-mapping studies have shown that the subpopulation of ventrally located $FGF10^{+}$ tanycytes mainly gives rise to parenchymal neurons of the arcuate and ventromedial nuclei (Haan et al. 2013), while $GLAST^{+}$ dorsal tanycytes generate astrocytes and, to a lesser extent, neurons (Robins et al. 2013). Moreover, adenovirus-mediated labeling of cells lining the third ventricle suggests that tanycytes of the adult rat hypothalamus generate neurons that are incorporated into the hypothalamic parenchyma, where they form synapses (Xu et al. 2005a). Finally, in vitro studies suggest that tanycytes exhibit neural stem/progenitor cell properties. When cells of the ependymal layer of the third ventricle of adult rats were labeled with the fluorescent marker DiI, isolated by fluorescence-activated cell sorting (FACS) and subjected to a neurosphere assay (see Sect. 6.2.3), ependymal cells, including tanycytes, were found to generate neurospheres that proliferated, self-renewed, and differentiated into all three neural cell types (Xu et al. 2005a). It should be noted that this protocol did not distinguish between tanycytes and more dorsal ciliated ependymal cells and thus did not confirm the tanycytic nature of neurosphere-forming cells. However, when the median eminence of adult transgenic mice expressing the green fluorescent protein (GFP) under the control of the nestin promoter was dissected out and cultured, fluorescent multipotent neurospheres that proliferated and self-renewed were obtained (Bennett et al. 2009). While nestin is expressed by both parenchymal progenitors and tanycytes in the mediobasal hypothalamus dorsal to the median eminence (Li et al. 2012), it is selectively expressed by tanycytes within the median eminence (Haan et al. 2013; Lee et al. 2012; Bennett et al. 2009) (Fig. 6.3a). These data hence suggest that the neurospheres were derived from nestin-expressing median eminence tanycytes. Moreover, we have shown that when primary cultures of rat tanycytes are transferred into neurosphere culture medium, they give rise to neurospheres that can be passaged and differentiated into neurons, astrocytes, and oligodendrocytes (Allet et al. 2006). Altogether, these data strongly argue in favor of tanycytes being

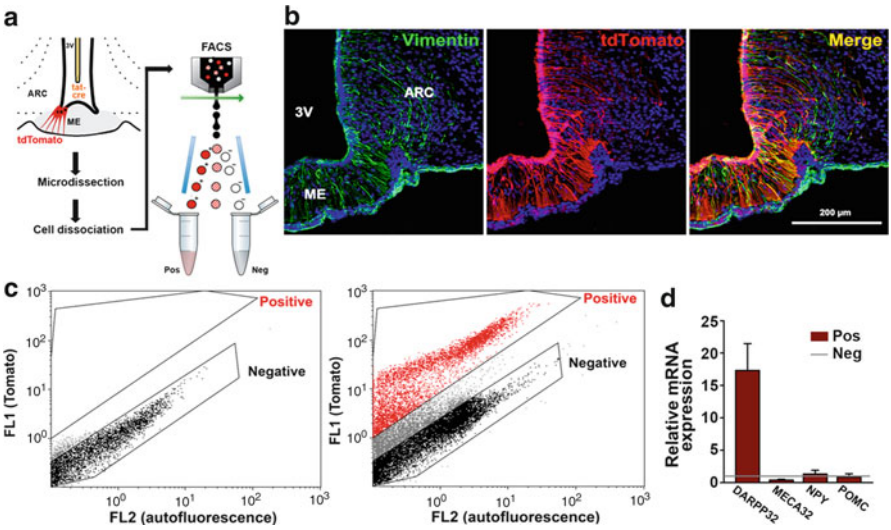

Fig. 6.4 Isolation of hypothalamic tanycytes using fluorescence-activated cell sorting (FACS) in adult mice. (**a**) Scheme depicting the sorting procedure. Briefly, the tat-cre recombinant protein is infused into the third ventricle of *tdTomato*$^{loxP/+}$ mice to induce recombination in cells lining the ventricle. After microdissection and dissociation of the median eminence, cells are separated by FACS into tdTomato-positive/fluorescent (Pos) and tdTomato-negative/nonfluorescent (Neg) cells. (**b**) Fluorescence microscopic images showing tomato expression (*red*) in vimentin (*green*)-immunoreactive tanycytes in *tdTomato*$^{loxP/+}$ mice that had been infused with the tat-cre recombinant protein. (**c**) FL1 vs. FL2 scatter plots showing the tdTomato-specific fluorescence (FL1) and autofluorescence (FL2) of cell suspensions from wild-type (*left panel*) and tdTomato-positive animals (*right panel*). The gating intervals were designed to specifically select the population of tdTomato-positive cells (*red*), excluding autofluorescent cells and a population of specifically negative cells (*black*). (**d**) Real-time PCR analysis of expression levels of various gene transcripts in tdTomato-positive cells relative to those in tdTomato-negative cells. Note that transcripts for the tanycytic marker DARPP32 are abundantly expressed in the positive fraction, while transcripts for the fenestrated endothelial cell marker MECA32 and for the neuronal cell markers NPY and POMC are not detected. *3V* third ventricle, *ARC* arcuate nucleus of the hypothalamus, *ME* median eminence. (Adapted from Langlet et al. (2013b) with permission from Elsevier)

hypothalamic neural stem cells. Nevertheless, additional experiments are needed to ascertain their "stemness." For example, the selective isolation of tanycytes using FACS-based procedures, such as the one developed in one of our laboratories (Fig. 6.4) (Langlet et al. 2013b), would be useful to specifically assess the stem cell-like properties of tanycytes.

Notably, tanycytes do not constitute a homogeneous population of cells. Indeed, distinct subpopulations have been recognized, along both the dorsoventral and the rostrocaudal extent of the third ventricle, with respect to their morphology, ultrastructure, molecular, and functional properties (Haan et al. 2013; Robins et al. 2013; Rodriguez et al. 2005; Mullier et al. 2010). The niche of proliferative and neurogenic tanycytes identified in the hypothalamus of early postnatal mice is composed

of tanycytes lining the ventral part of the third ventricle, which are particularly enriched in the caudal portion of the median eminence (Lee et al. 2012). The FGF10$^+$ neurogenic tanycytes identified later in life are also ventral tanycytes and are most abundant in the central regions of the mouse median eminence (Haan et al. 2013). Tanycytes located more dorsally also proliferate and self-renew in adult rodents (Xu et al. 2005a; Robins et al. 2013). In contrast to ventral tanycytes, they selectively respond in vivo to FGF2 (Xu et al. 2005a; Robins et al. 2013) and insulin-like growth factor I (IGF-I) (Perez-Martin et al. 2010) with strong proliferation. Moreover, the subpopulation of GLAST$^+$ dorsal tanycytes is preferentially gliogenic (Robins et al. 2013). Thus, ventral and dorsal tanycytes appear to differ with respect to their mitogenic response to growth factors and differentiation potential. Additional experiments, in particular specific lineage tracing of other subpopulations of tanycytes, are required to refine the characterization of the neural stem/progenitor cell properties of tanycytes along the ventricular lining.

6.3.3 Deciphering the Hypothalamic Stem Cell Lineage: Still a Long Way to Go

A number of elements are still needed before we can draw a comprehensive picture of the hypothalamic stem cell lineage. As detailed above, several arguments support the "stemness" of tanycytes. However, whether stem cells also reside in the hypothalamic parenchyma remains to be definitively proven. The higher proliferative activity seen in the parenchyma than in the tanycyte-bearing ependymal layer suggests that most dividing parenchymal cells are multipotent progenitors rather than stem cells. This assumption raises another question that of a possible lineage relationship between putative tanycytic stem cells and proliferative parenchymal cells. Interestingly, FGF10$^+$ tanycytes have been shown to give rise to parenchymal descendants, a subset of which continue to divide locally in the early postnatal and adult mouse hypothalamus (Haan et al. 2013). Whether these parenchymal descendants retain some stem cell properties or whether they represent a population of more committed neural progenitors is yet to be determined. Moreover, the expression of the neural stem/progenitor cell marker Sox2 is seen in both tanycytes and parenchymal cells (Li et al. 2012; Haan et al. 2013). Interestingly, two morphologically distinct populations of Sox2$^+$ neural stem cells have been described in the SGZ of the hippocampus: a radial type and a non-radial type, which belong to related lineages (Suh et al. 2007). It would be interesting to determine whether a similar relationship exists in the hypothalamus between radial Sox2$^+$ tanycytes and non-radial Sox2$^+$ stem/progenitor cells in the parenchyma. To address this question, the injection of a Sox2-promotor-driven lentiviral Cre recombinase into the third ventricle of ROSA_lox_STOP_lox_YFP mice could allow the fate mapping of Sox2$^+$ tanycytes to determine whether they give rise to parenchymal Sox2$^+$ cells. This strategy was used in the studies mentioned above to lineage-trace Sox2$^+$ progenitors in both the mediobasal hypothalamic parenchyma and hippocampus (Li et al. 2012;

Suh et al. 2007). Finally, the definitive identification of neural stem cells in the SVZ and SGZ was based on antimitotic treatments that depleted the proliferative progenitor pool while sparing quiescent neural stem cells (Doetsch et al. 1999a, b; Seri et al. 2001). This approach, combined with lineage-tracing studies, demonstrated that SVZ and SGZ astrocytes, which remained after antimitotic treatment, were capable of fully regenerating these neurogenic niches and hence were the neural stem cells (Doetsch et al. 1999a, b; Seri et al. 2001). Applying these experimental strategies to the study of the hypothalamus could provide significant insights into the identity of the hypothalamic stem cells and their lineage.

6.4 Modulators of Hypothalamic Neurogenesis

While constitutive neurogenesis in the hypothalamus is relatively slow under physiological conditions, it can be strongly stimulated by several physiological and pathological factors and situations. More recently, inhibitory cues, mostly related to altered metabolic conditions, have also been identified. These modulators act at many different steps of hypothalamic neurogenesis, from the maintenance of the stem cell pool to the specification of the newborn cell fate (Table 6.1).

6.4.1 Stimulatory Cues

Several neurotrophic and growth factors promote cell proliferation and neurogenesis in the adult rodent hypothalamus. Intracerebroventricular administration of *FGF2* selectively stimulates the proliferation of tanycytes located dorsal to the median eminence in adult rats (Xu et al. 2005a) and mice (Robins et al. 2013). *Brain-derived neurotrophic factor* (BDNF) is also a potent mitogen in the hypothalamus of adult rats. Notably, the extent of its proliferative effect is highly dependent on the experimental setting used, since continuous i.c.v. infusion for 12 days coupled with the i.c.v. delivery of BrdU reveals high levels of proliferation in the hypothalamic parenchyma (Pencea et al. 2001), while a single injection into the third ventricle coupled with the intraperitoneal administration of BrdU reveals only a weak mitogenic effect on ependymal cells of the third ventricle (Xu et al. 2005a). Moreover, the long-term i.c.v. infusion of BDNF doubles (from 21 to 41 %) the proportion of newborn cells that take on a neuronal fate without affecting the rate of astrogliogenesis. Interestingly, while BDNF also stimulates proliferation in the SVZ, it does not affect neuronal specification in this region (Pencea et al. 2001). Similar to BDNF, the neuropeptide *ciliary neurotrophic factor* (CNTF) delivered intracerebroventricularly is a strong inducer of cell proliferation throughout the hypothalamic parenchyma of adult mice and doubles the rate of newborn cells that differentiate into neurons without affecting glial specification (Kokoeva et al. 2005, 2007). Finally, *insulin-like growth factor I* (IGF-I) stimulates cell proliferation and

Table 6.1 Regulators of hypothalamic cell proliferation and neurogenesis

Regulatory factor	Animal model	Neural stem /progenitor cell pool		Neuronal differentiation[a]	Putative mechanism of action	References
		In vivo[b]	In vitro			
Growth and neurotrophic factors						
FGF2	Adult rat	+	+			Xu et al. (2005a)
	Adult mouse	+	+			Robins et al. (2013)
EGF	Adult rat	+ Weak	+ Weak			Xu et al. (2005a)
BDNF	Adult rat	+		+	Indirect effect since TrkB is not detected in BrdU-labeled cells	Pencea et al. (2001)
CNTF	Adult mouse	+		+	Possible direct effect since CNTF receptor is detected in BrdU-labeled cells	Kokoeva et al. (2005, 2007)
IGF-I	Adult rat	+		+	Possible direct effect since IGF-I receptor is detected in BrdU-labeled cells	Perez-Martin et al. (2010)
Hormones						
Leptin	Adult mouse	+	n.a.	n.a.	Probable indirect effect (see main text for details)	McNay et al. (2012)
Gonadal steroids	Pubertal rats	+				Ahmed et al. (2008)
	Adult prairie and meadow voles	n.a.				Fowler et al. (2005)
GnRH	Old mice	+				Zhang et al. (2013)
Neurodegenerative context						
Loss of ARC neurons	Young postnatal rat	+				Rodriguez et al. (2005)
Loss of AgRP neurons	Adult mouse	+		+ (New AgRP and POMC neurons)		Pierce and Xu (2010)

Others						
Heat	Adult rat	+		+		Matsuzaki et al. (2009)
Social environment	Adult prairie vole	+/–[c]				Fowler et al. (2002)
Photoperiod	Adult hamster	+/–[d]				Huang et al. (1998)
	Adult sheep	+/–[d]				Migaud et al. (2011)
High-fat diet						
Chronic HFD (2 months)	Adult mouse	–	+[e]	n.a.		McNay et al. (2012)
Chronic HFD (4 months)	Adult mouse	–	–	–	Local inflammation triggering activation of IKKβ/NF-κB signaling in hypothalamic neural stem/progenitor cells	Li et al. (2012)

+, stimulation; –, inhibition; n.a., not affected; absence of data, not examined

[a]Neuronal differentiation: percentage of newborn cells that take on a neuronal fate

[b]The size of the neural stem/progenitor cell pool in vivo, evaluated by BrdU incorporation or the expression of endogenous cell-cycle markers, is determined by the balance between cell proliferation and cell death. Notably, most studies do not distinguish between these two parameters, leading to the general interpretation that an increased number of BrdU-labeled cells reflect increased proliferation. However, the increased survival of newly divided cells may also contribute to this effect

[c]In adult female prairie voles, exposure to males significantly increases the number of BrdU-labeled cells in the hypothalamus compared to social isolation

[d]In both hamsters and sheep, two seasonal mammals, hypothalamic cell proliferation increases during short days compared to long days. Notably, the factors that mediate the effect of photoperiod or social environment on hypothalamic cell proliferation remain to be identified

[e]In vitro neurosphere assays reveal an expansion of the pool of hypothalamic neurosphere-forming cells (i.e., neural stemlike cells). This effect is counterbalanced by a decrease in the number of proliferative progenitor-like cells, as attested to by decreased BrdU incorporation in vivo after cumulative labeling over a short period of time, thereby leading to a global decrease in the pool of newly divided cells. It should be noted that the mode and duration of administration of growth/neurotrophic factors and hormones greatly varies between studies, from a single i.c.v. or peripheral injection to chronic delivery via implanted minipumps

increases the rate of neurogenesis in the adult rat hypothalamus (Perez-Martin et al. 2010). Notably, the mitogenic effect of IGF-I is most prominent in a distinct region of the third ventricle wall near the caudal hypothalamus. This region exhibits the structural hallmarks described for the two primary neurogenic niches of the SVZ and SGZ. Cell division in this region occurs in tanycytes and subependymal astrocytes, some of which exhibit an apical process contacting the cerebrospinal fluid and bearing one or two cilia: a structural feature of astrocytic stem cells in the SVZ (Doetsch et al. 1999b; Mirzadeh et al. 2008). This region also exhibits a particular abundance of basement membrane labyrinths in the subependymal zone, another structural characteristic of adult neurogenic niches (Alvarez-Buylla and Lim 2004). It remains to be determined whether similar structural features occur elsewhere in the third ventricle wall and, if so, whether they identify additional hypothalamic niches with a characteristic sensitivity to stimulation.

Hormones can also promote hypothalamic neurogenesis. *Leptin*, an adipocyte-derived hormone critically involved in the control of energy metabolism, acts primarily on specific populations of neurons in the mediobasal hypothalamus (Morton et al. 2006; Myers and Olson 2012; Williams and Elmquist 2012). Recently, leptin has been shown to regulate hypothalamic neurogenesis by acting specifically at the level of the stem cell pool (McNay et al. 2012). Indeed, mice genetically deficient in leptin (Zhang et al. 1994) exhibit a dramatic decrease in cell proliferation in vivo and in neurosphere-forming cells in vitro, while the specification of neuronal cell fate is unaffected. Conversely, the long-term subcutaneous infusion of leptin for 14 days in normal adult mice strongly increases the number of hypothalamic neurosphere-forming cells. It should be noted that any effect of leptin on hypothalamic stem cells may be indirect, since the short-term (7-day) i.c.v. infusion of leptin in normal mice fails to stimulate cell proliferation in vivo, and leptin application directly in vitro does not affect neurosphere formation (McNay et al. 2012). In young postnatal rats, *gonadal steroids* actively participate in the maintenance of the sexual dimorphism that characterizes several adult brain regions, including the hypothalamic nuclei AVPV and SDN, by stimulating not only programmed cell death (Waters and Simerly 2009) but also the addition of new cells in a sex- and region-dependent manner (Ahmed et al. 2008). However, in one study focusing on the ventromedial hypothalamus (VMH) of adult female prairie and meadow voles, estrogen treatment did not affect cell proliferation (Fowler et al. 2005). Whether gonadal steroids regulate the generation of new hypothalamic cells during adulthood in other rodent species and/or in other hypothalamic nuclei remains to be explored. Intriguingly, a recent study showed that *gonadotropin-releasing hormone* (GnRH), a neurohormone synthesized by a population of hypothalamic neurons that control the reproductive axis (Ojeda and Skinner 2006), stimulates cell proliferation in the hypothalamus of old mice (Zhang et al. 2013), an effect that may underlie the beneficial effects of GnRH treatment on ageing-related physiological and histological biomarkers, such as cognitive functions, muscle fiber size, and skin thickness (Zhang et al. 2013).

The selective neurodegeneration of hypothalamic neurons is a stimulatory signal for neurogenesis in the postnatal hypothalamus. The treatment of newborn rats with

monosodium glutamate (MSG) leads to massive neuronal death within the ARC of the hypothalamus (Hu et al. 1998), which is followed by an impressive, though partial, recovery of neuronal cell bodies and fibers within the next few weeks (Rodriguez et al. 2005). BrdU incorporation studies have revealed that MSG treatment induces a striking increase in cell proliferation and the generation of new neurons and glial cells in the ARC (Rodriguez et al. 2005). Interestingly, this proliferative activity is mostly seen in the subset of tanycytes that faces the ARC. Additional experiments are required to determine whether new ARC neurons originate from this specific population of tanycytes. Cell proliferation increases in the ARC of mutant mice suffering from progressive neurodegeneration of Agouti-related peptide (AgRP) neurons (Pierce and Xu 2010). AgRP neurons are key components of the circuit controlling energy balance within the ARC (Morton et al. 2006; Myers and Olson 2012; Williams and Elmquist 2012). Notably, this effect is specific to the ARC, since increased cell proliferation is not seen in the neighboring VMH, dorsomedial hypothalamus (DMH), or lateral hypothalamic area (LHA). Moreover, a subset of the newborn cells take on the identity of AgRP neurons, while others differentiate into POMC neurons, which work along with AgRP neurons within the ARC to regulate food intake and body weight (Morton et al. 2006; Myers and Olson 2012; Williams and Elmquist 2012). Thus, neurodegeneration seems to induce a local microenvironment that is permissive for cell proliferation and neurogenesis via inductive signals that remain to be identified.

Other regulators of adult hypothalamic proliferation and neurogenesis have also been identified, such as photoperiod (Huang et al. 1998; Migaud et al. 2011), social environment (Fowler et al. 2002), and heat exposure (Matsuzaki et al. 2009).

6.4.2 Inhibitory Cues

The effect of chronic high-fat diet (HFD) administration on postnatal hypothalamic neurogenesis in mice has been evaluated by several groups. When examining cell neurogenesis in the median eminence of prepubertal and young adult mice, Lee and colleagues reported that an HFD for 1 month increased the proportion of median eminence neurons that were newly born during the first week of HFD exposure (Lee et al. 2012). While this result primarily suggests that short-term HFD stimulates neurogenesis, it could alternatively reflect the decreased turnover of neurons born at the beginning of the HFD treatment. In agreement with this last assumption, two studies have clearly shown that chronic HFD administration is a potent inhibitor of postnatal hypothalamic neurogenesis in mice. Indeed, adult mice submitted to an HFD for several months exhibit a marked decrease in the generation of new cells within the ARC due to diminished cell proliferation and/or the increased apoptosis of newborn cells. These deficiencies ultimately lead to a reduction in the number of newborn neurons, including POMC and NPY neurons (McNay et al. 2012; Li et al. 2012). Notably, while these two studies agree that HFD impairs neurogenesis, they describe distinct underlying cellular mechanisms. McNay and colleagues showed

that adult mice subjected to an HFD for 2 months exhibit an expansion of the pool of hypothalamic neural stemlike cells, as manifest by increased number of neurosphere-forming cells in a neurosphere assay, while the population of actively dividing progenitor-like cells is depleted due, at least in part, to increased apoptosis. This decrease in the pool of progenitors, in the face of an unaltered rate of neuronal fate commitment, eventually leads to a reduction in the number of newborn neurons (McNay et al. 2012). In contrast, Li and colleagues reported that when adult mice are subjected to an HFD for 4 months, the pool of neural stem/progenitor cells is depleted due to impaired proliferation and survival and that the rate of neuronal differentiation is diminished (Li et al. 2012). Whether the different length of HFD feeding explains these discrepancies remains to be determined. Interestingly, in the McNay study, after 2.5 months of an HFD, the decreased generation of new neurons in the ARC is compensated for by the increased survival of embryo-born neurons, thereby maintaining the integrity but leading to the relative ageing of the hypothalamic energy-balance circuit (McNay et al. 2012). Notably, two studies have reported a decrease in the number of POMC neurons in the ARC after 8 months of an HFD (Li et al. 2012; Thaler et al. 2012), raising the possibility that compensatory mechanisms may be lost by a prolonged HFD regime. In the search for the molecular basis underlying the inhibitory effect of HFD on hypothalamic neurogenesis, Li and colleagues identified inflammation as a critical determinant of the neurogenesis deficit induced by long-term (4-month) HFD (Li et al. 2012). The results of this study suggest that in chronic HFD, microglia produce pro-inflammatory cytokines such as TNF-α and IL-1β that impair hypothalamic neural stem/progenitor cell proliferation, survival, and neuronal differentiation by activating IκB kinase β (IKKβ) and the downstream nuclear factor-κB (NF-κB). This deleterious paracrine action of microglia is then maintained by the establishment of an autocrine loop involving the IKKβ/NF-κB-induced production of TNF-α and IL-1β in hypothalamic stem/progenitor cells (Li et al. 2012). The observation that hypothalamic inflammation can be detected very early in rodents subjected to HFD (Thaler et al. 2012) raises the possibility that an inflammatory process may be a common feature of neurogenesis deficits induced by HFD, regardless of the exposure time.

The circulating metabolic signals that underlie the effect of overfeeding on hypothalamic neurogenesis and/or inflammation have not been identified. One possible scenario involves the induction of oxidative stress and mitochondrial dysfunction in hypothalamic neurons subjected to prolonged exposure to excessive amounts of circulating nutrients, leading to hypothalamic inflammation and subsequent neurogenesis deficits (Cai 2013). The deregulation of hormone levels may be an additional contributing factor. Because leptin has been shown to be involved in the maintenance of the hypothalamic stem cell pool (McNay et al. 2012), chronic HFD, which leads to leptin resistance (Enriori et al. 2007), may lead to depletion of the hypothalamic stem cell pool (Li et al. 2012). Whether hypothalamic neurogenesis is affected by other hormones whose levels are deregulated by chronic overfeeding, such as glucocorticoids, which negatively regulate the neuronal differentiation of progenitors and the maturation of newborn neurons in the hippocampus (Anacker et al. 2013; Fitzsimons et al. 2013), needs to be determined.

6.5 Functional Relevance of Hypothalamic Neurogenesis

With the demonstration that new neurons are continuously generated within the postnatal hypothalamus, there remain fundamental questions concerning their role in hypothalamic physiology and the putative involvement of deregulated neurogenesis in the development of hypothalamic pathologies.

6.5.1 Newborn Neurons Are Functionally Integrated into Hypothalamic Circuits

To determine whether neurogenesis is implicated in the regulation of hypothalamic function, it needs to be first demonstrated that newborn neurons are functionally embedded within the hypothalamic neuronal circuitry. That this is indeed the case is suggested by the demonstration of synapse formation between a newborn neuron and a resident neuron in the hypothalamic parenchyma of an adult rat (Xu et al. 2005a). Hypothalamic neurogenesis has been mostly studied in the mediobasal hypothalamus, particularly the ARC, where neurons sense and integrate metabolic signals linked to energy status, such as leptin (Morton et al. 2006; Myers and Olson 2012; Williams and Elmquist 2012). Leptin stimulates the phosphorylation and nuclear translocation of signal transducer and activator of transcription 3 (STAT3) in leptin-sensitive cells (Lee et al. 2012; Vaisse et al. 1996; Becskei et al. 2010; Caron et al. 2010). A subset of hypothalamic neurons born during the early postnatal period in the median eminence and during the prepubertal period in the ARC of mice show phosphorylation of STAT3 in response to leptin administration (Haan et al. 2013; Lee et al. 2012). Leptin signal transduction has also been demonstrated in a significant proportion of mouse mediobasal hypothalamic neurons born during adulthood, in response to CNTF administration (Kokoeva et al. 2005) or to the selective neurodegeneration of AgRP neurons (Pierce and Xu 2010). In addition, some newly generated neurons in the median eminence of young postnatal mice are responsive to acute fasting, as attested to by increased expression of the immediate early gene c-fos (Lee et al. 2012). The ability of newborn neurons to respond to exogenous leptin or fasting strongly suggests that they are functionally integrated into the energy-balance-regulating circuit. Nevertheless, incontrovertible demonstration that newborn neurons not only sense peripheral metabolic signals but also integrate and propagate these signals across the neuronal network requires electrophysiological evidence. Notably, the proof of concept for the functional integration of new neurons into postnatal hypothalamic circuitry has been provided by an electrophysiological study showing that immature hypothalamic neurons microtransplanted into the mediobasal hypothalamus of postnatal mice are synaptically integrated into the recipient hypothalamic circuitry. Moreover, the transplantation of normal hypothalamic neurons into leptin-receptor-deficient (db/db) obese mice partially restores leptin sensitivity and corrects metabolic deficits (Czupryn et al. 2011).

Although the electrophysiological examination of hypothalamic neurons born postnatally is not yet available, the pathophysiological consequences of altered hypothalamic neurogenesis (see below) argue in favor of a functional role for this process in the regulation of energy homeostasis.

6.5.2 *A Role for Hypothalamic Neurogenesis in the Control of Energy Homeostasis?*

As detailed above, some of the neurons born in the postnatal mediobasal hypothalamus express neuropeptides involved in energy-balance regulation, such as orexin, POMC, AgRP, and NPY, and exhibit responsiveness to peripheral metabolic inputs such as leptin and acute food deprivation, indicating their possible participation in the hypothalamic control of energy homeostasis. Studies conducted in the pathological context of metabolic disorders have shown that the manipulation of hypothalamic neurogenesis has functional consequences on food intake and body weight. Overfeeding alters neurogenesis in the median eminence of juvenile and young adult mice. In HFD-treated peripubertal mice, the targeted inhibition of neurogenesis in the ventrobasal hypothalamus, including the median eminence, by focal X-ray irradiation leads to reduced weight and fat mass gain and increased oxygen consumption, energy expenditure, and total activity compared to sham-irradiated HFD mice (Lee et al. 2012). These results show that postnatal median eminence neurogenesis is involved in the regulation of feeding and metabolism and suggest that its deregulation contributes to the mechanism by which alterations to the nutritional environment during early postnatal life exert long-term effects on metabolism (Simerly 2008). During adulthood, CNTF causes weight loss in obese rodents and humans, and this effect persists for weeks to months after the cessation of treatment (Kokoeva et al. 2005; Lambert et al. 2001; Ettinger et al. 2003), suggesting the long-term remodeling of hypothalamic circuits. In adult mice, chronic overfeeding negatively regulates hypothalamic neurogenesis (McNay et al. 2012; Li et al. 2012) (see Sect. 6.4.2), while CNTF strongly stimulates it, in both normal animals (Kokoeva et al. 2007) and those with diet-induced obesity (Kokoeva et al. 2005). In particular, CNTF promotes the generation of new NPY and POMC neurons in the ARC, thereby adding new components to the energy-balance circuitry. When CNTF-induced neurogenesis is blocked by the infusion of the antimitotic drug cytosine-β-D-arabinofuranoside (Ara-C), mice rapidly regain weight after the termination of CNTF treatment, showing that the stimulation of neurogenesis in the adult hypothalamus underlies the long-term weight-reducing effect of CNTF. Interestingly, some of the CNTF-induced newborn cells are responsive to exogenous leptin, and leptin is required for the persistence of the CNTF weight-loss effect since genetically leptin-deficient ob/ob mice regain weight after the cessation of CNTF treatment (Kokoeva et al. 2005). It could be speculated that the addition of new leptin-sensitive neurons due to CNTF treatment enhances the leptin-driven satiety signal, hence contributing to the sustained weight-loss effect of CNTF. Finally,

hypothalamic neurogenesis has been shown to be a compensatory mechanism for the maintenance of proper regulation of energy balance in a model of targeted neurodegeneration of AgRP neurons (Pierce and Xu 2010). While the acute ablation of AgRP neurons leads to severe anorexia and weight loss (Gropp et al. 2005; Luquet et al. 2005), progressive AgRP neuron degeneration does not impact food intake or body weight (Xu et al. 2005b). In transgenic mice undergoing the gradual loss of AgRP neurons over a period of several months, increased neurogenesis is seen in the ARC, and a subset of the newly generated cells differentiate into AgRP and POMC neurons. Moreover, a substantial proportion of these newborn cells are leptin sensitive. The inhibition of cell proliferation by the i.c.v. infusion of the antimitotic drug Ara-C significantly decreases food intake and body fat mass in mutant mice (Pierce and Xu 2010), suggesting that increased hypothalamic neurogenesis at least partially enables mice to escape the deleterious anorectic effects of AgRP neuron loss. Altogether, these data show that modulating postnatal hypothalamic neurogenesis can have an impact on feeding and energy metabolism.

The studies mentioned above have all been conducted in the pathological context of chronic overfeeding or targeted hypothalamic neurodegeneration, leaving unanswered the question as to the role of endogenous hypothalamic neurogenesis under normal physiological conditions, for example, in response to seasonal variations in food availability. Studies that identified stimulatory signals for hypothalamic neurogenesis in normal animals, such as growth factors and hormones (see Sect. 6.4.1; Table 6.1), did not evaluate the impact of enhanced neurogenesis on energy metabolism or other hypothalamus-dependent functions. When i.c.v. Ara-C infusion was used to assess the consequences of endogenous neurogenesis inhibition on metabolism in adult mice, Ara-C did not affect food intake, body weight, lean mass, or fat mass over a 4-week follow-up period (Pierce and Xu 2010). It is possible that the failure to detect an effect on metabolism may have been due to the short period (1 month) studied after the injection. This is suggested by another study in which a lentivirus-based strategy was used to specifically target hypothalamic neural stem/progenitor cells (Li et al. 2012). Adult mice that were injected in the mediobasal hypothalamus with a lentiviral vector to drive the constitutive activation of the proinflammatory IKKβ/NF-κB pathway in Sox2-expressing neural stem/progenitor cells exhibited a sharp decrease in hypothalamic proliferative activity, survival, and neuronal differentiation of newborn cells, leading to the depletion of the neural stem/progenitor pool and to the loss of ~10 % of POMC neurons 3 months postinjection. These animals developed glucose intolerance and hyperinsulinemia and showed increased food intake at 3 months, leading to the onset of severe obesity at 10 months, despite being given a normal diet (Li et al. 2012). This study shows that the selective impairment of hypothalamic neurogenesis in adult mice can have a major long-term deleterious impact on the regulation of metabolism and hence suggests that intact neurogenesis is required for the proper regulation of energy balance. Moreover, since the constitutive activation of IKKβ/NF-κB in hypothalamic neural stem/progenitor cells mimics the effects of chronic HFD (Li et al. 2012), these results suggest that the alteration of hypothalamic neurogenesis may be a critical early event in the onset of metabolic disorders such as obesity and diabetes in response to overfeeding.

6.6 Concluding Remarks

Postnatal neurogenesis in the mammalian brain is thought to be restricted to two regions, the SVZ/rostral migratory stream (which gives rise to new olfactory bulb neurons) and the SGZ of the hippocampal dentate gyrus. While the generation of new cells that express neuronal markers has been reported in other brain locations such as the neocortex and the striatum, these regions are still considered non-neurogenic due to the lack of reproducibility between studies and the frequent requirement for damaging conditions or pharmacological manipulation to observe neurogenesis (Migaud et al. 2010; Emsley et al. 2005; Gould 2007). On the other hand, since the first report of neurogenesis in the hypothalamus of adult hamsters 15 years ago (Huang et al. 1998), studies in a variety of animal models and experimental settings have been supporting the existence of constitutive neurogenesis and gliogenesis in the postnatal mammalian hypothalamus. In particular, the development of more sensitive assays of proliferation has been decisive in documenting the existence of this phenomenon, which occurs at lower levels in the hypothalamus than in the two primary neurogenic regions. Recent studies adding genetic fate mapping to classic BrdU-labeling protocols have conclusively demonstrated the constitutive generation of new neurons and glial cells in the hypothalamus throughout postnatal life. A number of regulatory factors, some of which also modulate neurogenesis in the SVZ and SGZ (Ming and Song 2005), have been identified. Interestingly, while the SVZ and SGZ are highly specialized in their neurogenic activity, giving rise to selected and restricted neuronal types (Alvarez-Buylla and Lim 2004; Ming and Song 2005; Lledo et al. 2006), the hypothalamus appears to be a source of more diverse newly generated cells, as the generation of several types of neuropeptide-containing neurons has been reported. Moreover, distinct hypothalamic locations seem to differ with respect to the fate specification of new cells, with CVOs being preferentially gliogenic, while mediobasal hypothalamic feeding centers such as the ARC appear to be mostly neurogenic. In-depth quantitative studies will be required to evaluate the extent and topography of the phenotypic diversity of newly generated cells across the hypothalamus.

While neurogenesis and gliogenesis have been consistently reported in the postnatal hypothalamus, a fundamental issue is whether or not this cellular plasticity is functionally relevant, especially because it occurs at low levels under basal conditions. Several studies have begun to address this question, with significant information coming from the field of energy homeostasis. The recent demonstration that the long-term, selective impairment of hypothalamic neural stem/progenitor cell proliferation, survival, and neuronal differentiation leads to the onset of metabolic disorders (Li et al. 2012) implicates deregulated neurogenesis in the etiology of hypothalamic pathologies. Notably, synaptic plasticity in energy-balance-regulating neuronal circuits is thought to be a critical adaptive mechanism to maintain

energy homeostasis in response to acute changes in the nutritional and hormonal environment (Zeltser et al. 2012). In this context, neurogenesis may represent another level of plasticity, providing these neuronal circuits with the ability to adapt to long-term changes.

A number of other questions still remain to be investigated. The identity, location, and lineage of hypothalamic stem cells are yet to be clarified. Given the heterogeneity in the phenotype of newborn cells, do several neural stem/progenitor populations exist? What are the molecular signals that control the maintenance of this hypothalamic germinal activity; the proliferation, survival, and fate specification of stem cells; and the differentiation and integration of newborn neural cells? If newly generated neurons are synaptically integrated into hypothalamic neuronal circuits, as seems to be the case, do they exhibit specific electrical properties that differ from those of older neurons, as has been suggested for new olfactory bulb and dentate gyrus neurons (Lledo et al. 2006)? In addition, the function of endogenous hypothalamic neurogenesis remains, for the most part, a mystery. As elaborated above, the role of hypothalamic neurogenesis in the control of metabolism has mainly been explored in a pathological context. Determining whether or how this process regulates energy balance under normal physiological conditions awaits future explorative studies. Importantly, since the pioneering study of Kokoeva and colleagues implicating hypothalamic neurogenesis in the control of food intake and body weight (Kokoeva et al. 2005), attention has been focused on the control of metabolism. However, this is only one of the many critical physiological functions regulated by the hypothalamus. The orchestration of the reproductive axis is another key hypothalamic function requiring a high degree of structural plasticity between GnRH neurons and their neuronal and non-neuronal partners (Prevot et al. 2010; Ojeda et al. 2008, 2010). The detection of neurogenesis and gliogenesis in hypothalamic regions controlling GnRH function, such as the AVPV, the OVLT, and the median eminence (Ahmed et al. 2008; Lee et al. 2012; Bennett et al. 2009; Hourai and Miyata 2013; Morita et al. 2013), raises the possibility that this process might also participate in mechanisms of plasticity involved in the neuroendocrine regulation of reproduction. Finally, while hypothalamic neurogenesis and gliogenesis have now been described in several mammalian species, it remains to be determined whether these phenomena exist in nonhuman primates and humans.

In short, in spite of its small size, the hypothalamus offers a vast and seemingly limitless area of investigation into the mechanisms of plasticity subserving the neural regulation of vital physiological functions.

Acknowledgments This research was supported by the Institut National de la Santé et de la Recherche Médicale (Inserm, France) Grant U837, the Fondation pour la Recherche Médicale (Equipe FRM 2005 and DEQ20130326524), the Agence Nationale de la Recherche (ANR) grant ANR-09-BLAN-0267, the Université Lille 2, and the IFR114, in addition to a grant from the National Science Foundation (NSF, grant IOS1121691) to SRO. We thank Dr. S. Rasika for the editing of our manuscript.

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Chapter 7
Regenerative Potential of NG2 Cells

Jean-Marie Mangin

Abbreviations

ALS	Amyotrophic lateral sclerosis
AMPA	2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
Ara-C	Arabinofuranosyl cytidine
BAC	Bacterial artificial chromosome
BMP	Bone morphogenetic protein
BrdU	5-Bromo-20-deoxyuridine
CNP	2′,3′-Cyclic-nucleotide 3′-phosphodiesterase
CNS	Central nervous system
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GABA	Gamma-aminobutyric acid
GalC	Galactosylceramidase
HDAC	Histone deacetylase
hIPSC	Human induced pluripotent stem cells
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NG2	Nerve/glial antigen-2

J.-M. Mangin (✉)
French Institute of Health and Medical Research, U952,
Pierre-and-Marie-Curie University, Paris, France
e-mail: jean-marie.mangin@inserm.fr

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3_7,

OPC	Oligodendrocyte progenitor cell
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PEDF	Pigment epithelium-derived factor
PI_3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLP	Myelin proteolipid protein
pMN	Progenitor of motor neurons
Shh	Sonic hedgehog
SVZ	Subventricular zone
VZ	Ventricular zone

7.1 The Self-Regenerative Ability of NG2 Cells

In the central nervous, NG2 cells are not only identified by their expression of the nerve/glial antigen-2 (NG2), a chondroitin sulfate proteoglycan (Stallcup 1981; Dawson et al. 2000), but also through the presence of other specific markers such as the ganglioside antigen recognized by the A2B5 antibody (Wolswijk and Noble 1989) and the platelet-derived growth factor (PDGF) receptor α (PDGFRα; Redwine and Armstrong 1998). These additional markers are often necessary to differentiate NG2 glial cells from the other cell type expressing NG2 in the nervous system; the pericytes associated to blood capillaries.

7.1.1 Origin of NG2 Cells

7.1.1.1 Embryonic Origin

The first NG2 cells arise during development from neuroepithelial progenitor cells in the ventral neural tube around embryonic day 12 (E12) in mice (Rowitch 2004; Richardson et al. 2006). In the spinal cord, most NG2 cells arise from two concomitant ventral domains: the pMN domain expressing the transcription factors olig1 and olig2 and the ventrally adjacent p3 domain characterized by the expression of Nkx.2.2 (Tekki-Kessaris et al. 2001; Kessaris et al. 2006). Both of these ventral domains are specified in response to the morphogen Sonic Hedgehog (Shh), secreted by the floor plate of the neural tube (Orentas et al. 1999; Lu et al. 2000; Richardson et al. 2006). A recent study suggests that fibroblast growth factor (FGF) signaling could also have an important role in specifying early embryonic NG2 cells, since deletion of either FGF receptor 1 or 2 using conditional KO mice is sufficient to block their specification (Furusho et al. 2011). The earliest OPCs, characterized by the specific expression of PDGFRα, can be detected in the ventral ventricular zone (VZ) as soon as E12 in mice. It has been reported that expression of the proteoglycan NG2 is delayed in early OPCs and it is usually detected once

OPCs have migrated out of the ventral VZ around E14 (Nishiyama et al. 1996; Zhu et al. 2011).

Although most NG2 cells originate from the ventral domains, a second wave of OPCs has been shown to arise around E15 in a Shh-independent manner from dorsal regions, notably from a dorsal progenitor domain expressing the transcription factor Dbx1 (Cai et al. 2005; Vallstedt et al. 2005; Fogarty et al. 2005). It has been shown that ventral and dorsal NG2 cells colonize different part of the CNS but genetic depletion of one source is compensated for by the others (Kessaris et al. 2006). Dorsally and ventrally derived NG2 cells have been shown to exhibit similar electrical properties (Tripathi et al. 2011). Therefore, these domains appear mostly redundant, and there is no evidence that NG2 cells generated from different domains differ in any significant way.

After the first pool of embryonic NG2 cells have been produced from their precursor domains in the VZ, they colonize the whole CNS during the next 2 weeks by following a ventrodorsal progression. This colonization process occurs through a combination of long distance migration and extensive secondary proliferation. NG2 cells reach their highest density during the first postnatal week and from that point they appear distributed at an almost uniform density in most gray and white matter areas (Nishiyama et al. 1996; Dimou et al. 2008; Rivers et al. 2008). As the brain grows to its adult size and myelination occurs, NG2 cell density will tend to slightly decrease before reaching its adult level (Nishiyama et al. 1996; Velez-Fort et al. 2009). However, NG2 cell distribution is never perfectly uniform, and careful examination has shown that these cells tend to preferentially accumulate at the border between distinct gray matter nuclei and could therefore participate in establishing certain glial boundaries in the CNS (Steindler 1993; Mangin and Gallo 2011; Mangin et al. 2012). This specific accumulation is regulated by neuronal activity and could participate in the preferential localization of white matter tracts at the boundaries between functionally distinct brain structures (Mangin et al. 2012).

7.1.1.2 Adult Origin

While most NG2 cells produced during the life of an adult brain are generated through the local division of surrounding NG2 cells (Kang et al. 2010; Robins et al. 2013; Hughes et al. 2013), they can also be generated de novo from the adult neural stem cells present in specialized niches such as the subventricular zone located along the lateral ventricles in the forebrain (Nait-Oumesmar 1999; Menn et al. 2006) and the dentate gyrus (Jessberger et al. 2008). The mechanisms involved in specifying NG2 cells from adult SVZ progenitors appear somewhat distinct from the one reported during embryonic specification. These differences may notably be explained by the fact that the production of NG2 cells from adult stem cells is not a constitutive process (Menn et al. 2006) but is largely dependent on a demyelination event occurring in areas surrounding the niche (Nait-Oumesmar et al. 1999; Picard-Riera et al. 2002; Menn et al. 2006). In a normal context, adult neural stem cells will mostly generate neurons—i.e., granule cells in the dentate gyrus and interneurons in

the SVZ. Therefore, the generation of NG2 cells from adult neural stem cells requires a switch from a default neuronal fate toward an oligodendrocyte fate. One of the factors allowing this fate switch is the bone morphogenetic protein (BMP) antagonist chordin, which has been shown to be upregulated after experimental demyelination in the corpus callosum (Jablonska et al. 2010). EGF signaling also appears to play a central role in redirecting the fate of SVZ precursors toward an NG2 cell phenotype and the generation of oligodendrocytes (Aguirre et al. 2007; Aguirre and Gallo 2007; Gonzalez-Perez et al. 2009). Finally, generation of NG2 cells from SVZ precursors, as well as their maturation into oligodendrocytes, has been shown to be promoted by the pigment epithelium-derived factor (PEDF) (Sohn et al. 2012), which is known to be secreted by adult SVZ endothelial and ependymal cells (Ramirez-Castillejo et al. 2006). In most cases, NG2 cells generated from adult SVZ progenitors remain in the immediate surrounding tissue, and SVZ-derived oligodendrocytes have only been reported in the corpus callosum, the fimbria, and the striatum, even in a mouse model of experimental autoimmune encephalomyelitis where a global demyelination occurs (Picard-Riera et al. 2002). In all other brain areas, NG2 cell replacement appears to rely exclusively on self-regeneration through symmetrical division of NG2 cells (Robins et al. 2013; Hughes et al. 2013).

7.1.2 NG2 Cell Proliferation

Lineage tracing and 5-bromo-20-deoxyuridine (BrdU) labeling in vivo have shown that NG2 cells divide throughout the adult rodent brain and represent the largest population of proliferative cells in adult brains (Dawson et al. 2003; Kang et al. 2010). At all ages, it has been shown that at least half of all NG2 cells are actively cycling (Kukley et al. 2008; Psachoulia et al. 2009), although their division rates appear to considerably slow down during development from a cycle rate of around 2 days in 1-week-old mice to a cycle rate of 70 days in 8-month-old mice (Psachoulia et al. 2009). The rate of NG2 cell division has been shown to depend on their location. It is slower in gray matter areas than in white matter tracts (Psachoulia et al. 2009; Young et al. 2013). Differences have also been observed between white matter areas, NG2 cell cycle being faster in partially myelinated axon tracts such as the corpus callosum than in fully myelinated tracts such as the optic nerve. Because only half of all NG2 cells in the adult brain appeared to incorporate BrdU, even when delivering it for several weeks to months (Dimou et al. 2008; Rivers et al. 2008; Psachoulia et al. 2009), it had been proposed that the pools of cycling and quiescent NG2 cells represented two distinct populations (Bouslama-Oueghlani et al. 2005; Dimou et al. 2008; Rivers et al. 2008; Psachoulia et al. 2009). However, several recent studies have reported that virtually all NG2 cells retain mitotic activity (Kang et al. 2010; Clarke et al. 2012; Young et al. 2013).

It is currently unclear whether the reduction in proliferation rate occurring during development reflects an intrinsic reduction of NG2 cell regenerative potential. However, it has been shown that the rate of NG2 cell proliferation is at least

partially correlated to their rate of differentiation into oligodendrocytes (Young et al. 2013; Hughes et al. 2013). Therefore, since most myelination occurs early during development, the reduction in NG2 cell proliferation in adult brain may simply reflect a decrease in the need for new myelin and oligodendrocytes. Indeed, NG2 cells have been shown to quickly proliferate in response to injuries or other conditions leading to their local depletion. Accordingly, after a demyelinating event, NG2 cell density tends to be depleted through local differentiation into oligodendrocytes, and sites of active remyelination will often exhibit higher rates of NG2 cell proliferation than the surrounding tissue (Carroll and Jennings 1994; Redwine and Armstrong 1998). The ability of NG2 cells to regenerate themselves through proliferation has been well illustrated by the laboratory of William Blakemore when studying the response of NG2 cells to a local depletion induced by X-ray irradiation in the adult rat spinal cord (Hinks et al. 2001; Chari and Blakemore 2002; Chari et al. 2006; Irvine and Blakemore 2007). Using this model, they demonstrated that adult NG2 cells have a considerable potential for self-renewal and that endogenous NG2 cells could recolonize adjacent OPC-depleted areas at a rate of 500 μm/week. They also found that NG2 cell density remained stable in surrounding areas, demonstrating that NG2 cells in spared areas would both maintain their local density and provide new NG2 cells to the depleted area. A recent study using live imaging in the adult mouse cortex has shown that the maintenance of population homeostasis in NG2 cells is a very dynamic process (Hughes et al. 2013). NG2 cells constantly move in relation to each other, extend filopodia to survey their environment and maintain distinct territory through dynamic self-avoidance. The loss of even a single NG2 cell through cell death or differentiation is usually compensated by proliferation and migration of neighboring NG2 cells, allowing NG2 cells to maintain a constant density (Hughes et al. 2013). However, the fact that many NG2 cells are slowly cycling and that all NG2 cells tend to divide in response to massive cell loss appears to make them extremely sensitive to mitotic blockers such as ara-C (Robins et al. 2013). This sensitivity may contribute to the detrimental consequence of chemotherapy on brain function, the so-called chemo fog, since decrease in NG2 cell proliferation and demyelination has been reported in response to various chemotherapeutic agents (Wigmore 2013).

Moreover, the potential of NG2 cells to regenerate is not infinite and can apparently exhaust itself, a phenomenon that could partially explain the lack of remyelination at the latest stages of the chronic demyelinating disease multiple sclerosis (MS) (Franklin and Ffrench-Constant 2008). Therefore, a better understanding of the factors regulating NG2 cell proliferation appears crucial to develop strategies aiming at restoring or enhancing the regenerative potential of NG2 cells in these diseases.

7.1.2.1 Role of Growth Factors in NG2 Cell Proliferation

The predominant and most specific mitogen known to control and regulate NG2 cell proliferation is the platelet-derived growth factor (PDGF), which acts on NG2 cells through the PDGF receptor α (PDGFRα), a tyrosine kinase receptor

(Richardson et al. 1988; Calver et al. 1998; Fruttiger et al. 1999). While PDGFRα activation is known to regulate NG2 cell proliferation, migration, and differentiation, its promitotic influence appears to be more specifically mediated via the PI_3K pathway (Ebner et al. 2000). In vitro studies have also shown that the fibroblast growth factor (FGF) has a strong promitotic effect on NG2 cells by acting through the FGF receptors 1 and 2 (Bogler et al. 1990; McKinnon et al. 1990; Wolswijk and Noble 1992; Frost et al. 2003; Fortin et al. 2005). However, recent studies using conditional KO of FGFR1 and FGFR2 suggest that FGF may not be directly involved in controlling NG2 cell proliferation in vivo but is rather required for the initial specification of NG2 cells at embryonic stages (Furusho et al. 2011). Finally, epidermal growth factor (EGF) has also been shown to promote NG2 cell proliferation through the EGF receptor (EGFR) and can lead to hyperplasia when constitutively expressing EGFR in NG2 cells (Aguirre et al. 2007; Ivkovic et al. 2008).

Since all these mitogens increase NG2 cell proliferation, they could be used to enhance and promote NG2 cell regeneration in vivo. Among all these mitogens, PDGF appears as a particularly attractive target since it has an important and relatively specific influence on NG2 cell proliferation. Indeed, the only other cells in the brain known to express a receptor to PDGF are the pericytes expressing the PDGF receptor β. However, while these growth factors have a powerful promitotic effect, it has also been shown that over-activation of growth factor-associated pathways can lead to hyperplasia and/or tumor formation. In fact, a number of studies suggest that NG2 cells could be the cell of origin in oligodendrogliomas (Briançon-Marjollet et al. 2010; Persson et al. 2010; Sugiarto et al. 2011) and low-grade human gliomas (Liu et al. 2011) and could form malignant gliomas in rodent in response to PDGF (Assanah et al. 2006).

The proteoglycan NG2 itself has been shown to regulate NG2 cell proliferation. By using an NG2 knockout mouse, Kucharova and Stallcup have shown that an absence of the proteoglycan NG2 leads to a decrease in proliferation rate and a subnormal density of OPCs in the cerebellum (Kucharova and Stallcup 2010). The influence of NG2 on cell proliferation may be explained by its ability to directly bind PDGF and FGF (Goretzki et al. 1999). Moreover, the asymmetric distribution of the proteoglycan NG2 in dividing cells appears responsible for an asymmetric distribution of FGFR in daughter cells during asymmetric division—i.e., division leading to progeny of different fates than NG2 cell, such as a pre-myelinating oligodendrocyte (Sugiarto et al. 2011). Loss of asymmetric division can lead to increased NG2 cell renewal and participate in tumor formation (Sugiarto et al. 2011). Therefore, it may be possible to promote NG2 cell differentiation and possibly reduce tumor formation, for example, in response to growth factors (Knoblich 2010), by directly manipulating NG2 expression.

7.1.2.2 Activity-Dependent Regulation of NG2 Cell Proliferation

As for many other cell types (Blackiston et al. 2009), NG2 cell proliferation has been shown to be regulated by the activity of a number of voltage-gated ion

channels as well as by ligand-gated ion channels. Voltage-gated potassium (K^+) channels have been shown to play a particularly significant role in regulating NG2 cell proliferation in vitro (Gallo et al. 1996; Knutson et al. 1997; Ghiani et al. 1999). For example, overexpression of potassium channel subunit Kv1.3 or Kv1.4 can promote NG2 cell proliferation in the absence of mitogens, whereas Kv1.6 overexpression inhibits mitogen-induced cell cycle progression (Vautier et al. 2004). However, although there is evidence that the proliferation and differentiation status of endogenous NG2 cells is correlated with changes in voltage-gated potassium current amplitude (Chittajallu et al. 2005; Kukley et al. 2010), evidence is lacking to support the influence of voltage-gated K^+ channels on the behavior of endogenous NG2 cells in vivo.

NG2 cell are also known to express ionotropic receptors to glutamate and GABA, and their activation has been shown to influence NG2 cell proliferation and differentiation in culture (Patneau et al. 1994; Gallo et al. 1996; Yuan et al. 1998). However, the exact influence of these neurotransmitters in vivo remains unclear. It has been shown that virtually all NG2 cells receive glutamatergic synapses and are also frequently contacted by GABAergic synapses from neurons (Bergles et al. 2000; Lin and Bergles 2004; Frohlich et al. 2011). NG2 cells conserved these synaptic inputs while dividing (Kukley et al. 2008; Ge et al. 2009; Tanaka et al. 2009) but lose them as soon they differentiate. Therefore, it seems likely that these synapses could regulate NG2 cell proliferation. Indeed, a recent study suggests that glutamatergic synapses between neurons and NG2 cells could mediate the influence of early sensory experience on NG2 cell proliferation and distribution in the mouse somatosensory cortex (Mangin et al. 2012).

7.1.3 NG2 Cell Migratory Potential

The capacity of NG2 cells to regenerate themselves is not only dependent on their ability to proliferate in response to cell loss but also to migrate into depleted areas. During embryonic and early postnatal development, at a time when NG2 cell density has not yet reach its peak adult density, NG2 cells have been shown to migrate long distances in order to colonize large territories (Sugimoto et al. 2001; Aguirre and Gallo 2004; Barres 2008; Cayre et al. 2009). In adult tissue, NG2 cells have recently been shown to constantly move in relation to each other (Hughes et al. 2013), but the distances traveled tend to remain limited in a normal context. However, as observed for proliferation, adult NG2 cells possess impressive latent migratory abilities and can efficiently migrate to repopulate, and if necessary remyelinate, large areas of the brain (Chari and Blakemore 2002; Foote and Blakemore 2005; Etxeberria et al. 2010; Robbins 2013). On the other hand, defects in migration can be responsible for delayed or absence of myelination (Cayre et al. 2009). Moreover, since NG2 can act as cell of origin in brain tumors, the "hijacking" of their unique migratory potential could participate to the invasiveness of certain tumors.

NG2 cell migration has been shown to be influenced both by soluble signaling factors and by cell–cell contact interactions (de Castro and Bribián 2005; Bradl and Lassmann 2010).

Secreted factors include growth factors such as PDGF and FGF (Armstrong et al. 1990; Milner et al. 1997; Fortin et al. 2005; Vora et al. 2011) and the hepatocyte growth factor (Ohya et al. 2007). Interestingly, growth factors appear to regulate NG2 cell proliferation and migration through distinct intracellular pathways. For example, the effect of PDGF on cell migration may be specifically dependent on ERK1/2 signaling (Frost et al. 2009). Several secreted guidance cues, such as netrin (Jarjour et al. 2003), semaphorins (Spassky et al. 2002), and chemokines (Tsai et al. 2002; Vora et al. 2012), have also been shown to influence the dispersion of NG2 cell during development. In the adult brain, there is evidence that semaphorins are involved in attracting NG2 cells to demyelinated areas (Piaton et al. 2011) while netrins and chemokines have been found to promote remyelination (Jaerve and Muller 2012; He and Lu 2013).

Cell–cell direct interactions between axons and NG2 cells appear to play a critical role in OPC migration (de Castro and Bribián 2005). During development, NG2 cells preferentially migrate along axonal tracts, a phenomenon that has been particularly well studied in the optic nerve (Small et al. 1987; Ono et al. 1999; Spassky et al. 2002). Many contact-mediated mechanisms have been identified including extracellular matrix proteins, *N*-cadherins, neuregulins, ephrins, and integrins (for review see de Castro and Bribián 2005). Axon-NG2 cell glutamatergic synapses could also be involved in regulating NG2 cell migration. Indeed, it has been shown that glutamate can influence the migration of NG2 cells through the activation of AMPA receptor (AMPAR) and the modulation of integrins (Gudz et al. 2006).

Moreover, NG2 cells express factors that can regulate their own migration. For example, the proteoglycan NG2 itself can play a role in NG2 cell directional migration (Binamé et al. 2013). Such factors may be involved in mechanisms of self-repulsion observed between NG2 cells (Hughes et al. 2013). In fact, dynamic self-repulsion could be the major factor in determining NG2 cell migration speed and direction in many contexts. Indeed, NG2 cells exhibit their highest migratory potential when encountering a territory devoid of other NG2 cells, either during development when new territories are available for colonization (Cayre et al. 2009) or after a territory has been freed from NG2 cells, either experimentally or because of an injury (Chari and Blakemore 2002; Blakemore and Irvine 2008). In accordance, transplanted NG2 cells usually exhibit a poor ability to migrate and populate large areas, except when endogenous NG2 cells have been depleted beforehand (Blakemore and Irvine 2008).

7.2 NG2 Cells and Oligodendrocyte Regeneration

7.2.1 Oligodendrocyte Generation from NG2 Cells

To generate myelinating oligodendrocytes, NG2 cells have to undergo a number of transformations, which have been relatively well defined (Zuchero and Barres 2013). The earliest marker expressed by NG2 cells when differentiating into oligodendrocytes

is the antigen O4 (Wolswijk 1998). O4 is one of the few markers that can be found co-expressed with the proteoglycan NG2 and PDGFRα before these markers disappear as differentiation progresses. As they acquire a pre-myelinating phenotype, NG2 cells also exhibit an important reorganization of their cell processes, which start aligning with surrounding axons. At this stage, they have lost NG2 expression and acquired markers of myelinating oligodendrocytes such as GalC and CNP (Wolswijk 1998). Then, as they ensheath axons with myelin, they upregulate myelin-associated proteins such as PLP, MBP, MAG, and MOG. Once generated, new oligodendrocytes have a limited amount of time to find and start myelinating axons and they become unable to myelinate new axons after they mature (Watkins et al. 2008). Moreover, NG2 cells appear to lose their ability to proliferate and become irreversibly postmitotic as soon as they initiate their differentiation into oligodendrocytes. Unlike Schwann cells in the peripheral nervous system (Mirsky et al. 2008), oligodendrocytes seem unable to reenter the cell cycle by a process of dedifferentiation (Keirstead and Blakemore 1999). It means that if a local pool of NG2 cells were to be exhausted through differentiation, the depleted area would lose all ability to regenerate myelin unless recolonization from surrounding tissue can occur. As a safety mechanism, the myelogenic potential of NG2 cells appears to depend on their density. Indeed, it has been shown that the ability of NG2 cells to generate oligodendrocytes in vitro requires them to first reach a critical density (Rosenberg et al. 2008). It is still unclear whether such a mechanism plays a significant role in vivo, but it has been observed that NG2 cells only start differentiating after they reach a maximum and almost uniform density during development (Nishiyama et al. 1996; Velez-Fort et al. 2009) and myelination tends to preferentially occur at a site of high NG2 cell density (Mangin et al. 2012). Interdependence between NG2 cell differentiation, proliferation, and migration is supported by the fact that these processes are often regulated by similar factors and mechanisms (Chong and Chan 2010; Zuchero and Barres 2013).

However, a number of factors have also been shown to specifically regulate NG2 cell differentiation. For example, deletion of the transcription factor Olig1 induces a defect in oligodendrocyte specification without interfering with the normal generation of NG2 cells during development (Xin et al. 2005). Olig1 has notably been shown to upregulate several myelin genes, including MBP, PLP, and MAG (Xin et al. 2005; Li et al. 2007). Moreover, remyelination is impaired in the Olig1 knockout mouse (Arnett et al. 2004). Several members of the transcription factor family Sox (Chew and Gallo 2009) such as Sox 10 (Stolt et al. 2002) and Sox 17 (Sohn et al. 2006; Ming et al. 2013; Moll et al. 2013) are known to regulate NG2 cell myelogenic potential. The transcription factors myelin gene regulatory factor (MYRF/gm98) and Zfp191 have also been recently identified as key regulators of myelin gene expression (Emery et al. 2009; Howng et al. 2010), and MYRF is required in adult oligodendrocytes to maintain their identity (Koenning et al. 2012).

Several recent studies have also shown that the ability of NG2 cells to differentiate into oligodendrocytes is influenced by epigenetic regulation such as histone acetylation. Indeed, activation of histone deacetylases (HDAC) is required for the downregulation of oligodendrocyte differentiation inhibitors, and it has been shown

that the administration of HDAC inhibitors impairs remyelination after cuprizone treatment (Shen et al. 2008). This epigenetic control could be influenced through the Wnt signaling pathway (Li and Richardson 2009). Thus, the Wnt pathways could provide a promising extracellular route to manipulate the epigenetic regulation of NG2 cell differentiation (Chong and Chan 2010).

7.2.2 NG2 Cells and Remyelination

Loss or dysfunction of oligodendrocytes and myelin is observed in a large number of brain pathologies, not only "classical" demyelinating diseases such as MS and congenital leukodystrophies but also brain and spinal cord injuries, age-related afflictions such as Alzheimer's disease, and various other pathologies from amyotrophic lateral sclerosis (ALS) to schizophrenia (Goldman et al. 2012). In the early stages of a demyelinating disease such as MS, demyelinated axons are usually effectively remyelinated, leading to remission. Indeed, endogenous NG2 cells can often fully regenerate lost myelin after a demyelinating episode, restoring saltatory conduction and allowing a complete functional recovery (Smith et al. 1979; Jeffery and Blakemore 1997; Liebetanz and Merkler 2006). The only detectable differences between native and remyelinated axons appear to be shorter internodes and thinner myelin, a phenomenon visible in MS patients as shadow plaques (Ludwin and Maitland 1984). However, as the disease progresses and with age, the remyelination efficiency decreases and chronic demyelination starts to be observed. Over time, the chronic loss of myelin not only disturbs transmission of nervous influx but also leads to axonal degeneration (Edgar and Nave 2009).

Before axon degeneration occurs, failure to regenerate myelin cannot only be due to a failure in recruiting a sufficient pool of NG2 cells into the lesion, but can also result from a failure of NG2 cells to differentiate into oligodendrocytes. Deficiencies in NG2 cell proliferation and migration have been inferred from the study of non-remyelinating lesions in MS patient where NG2 cells are found at a lower density than in normal tissue or are completely absent (Wolswijk 1998, 2000; Chang et al. 2000, 2002). The fact that NG2 cells can still be found in some non-remyelinating lesions but fail to generate oligodendrocyte suggests that demyelinating diseases can involve defects in NG2 cell differentiation. However, proliferation, migration, and differentiation appear tightly linked in order to achieve a homeostatic regulation of NG2 cell density (Hughes et al. 2013), and an absence of differentiation of NG2 cells remaining in the lesion may not necessarily result from an intrinsic inability to differentiate but could reflect a trade-off between the need for myelin regeneration and the need to preserve a minimal reserve of NG2 cells. Indeed, NG2 cells being the only source of new oligodendrocytes, forcing their differentiation would lead to a definitive loss of remyelination potential.

By using animal models of acute demyelination that allows successful remyelination such as the injection of toxic compounds (ethidium bromide, lysolecithin, etc.), it has been possible to identify the major steps involved in successful

remyelination (Franklin and Kotter 2008). A first and important step is an inflammatory reaction leading to the activation of microglia and astrocytes, which start releasing numerous inflammatory factors that activate local NG2 cells. Activated NG2 cells become then sensitized to chemoattractants and promitotic factors released by microglia and astrocytes, such as PDGF and FGF (Woodruff et al. 2004; Murtie et al. 2005). NG2 cells localized in the lesions will start proliferating and exhibit morphological changes associated with the expression of oligodendrocyte markers such as CNP (Reynolds et al. 2002). It has been shown that NG2 cell differentiation depends on the persistence of inflammation, and remyelination can fail if the recruitment of NG2 cell to the lesion does not occur in a timely fashion (Blakemore and Irvine 2008). Accordingly, induction of an inflammatory response can stimulate remyelination by endogenous NG2 cells in cases of chronic demyelination (Foote and Blakemore 2005). Most of the genes activated in NG2 cell-dependent remyelination are the same as the ones activated during developmental myelination (Fancy et al. 2011). However, a number of signaling pathways may be specifically activated during remyelination. For example, signaling through the retinoid X receptor gamma has been involved in NG2 cell differentiation and remyelination, despite the fact that it is not involved during developmental myelination (Huang et al. 2011).

Aging has a significantly influence on the ability of NG2 cell to remyelinate a lesion. This reduced remyelination potential within aging NG2 cells is both due to changes in extrinsic factors regulating remyelination (Hinks and Franklin 2000) and epigenetic changes (Shen et al. 2008).

The causes of remyelination failure in MS and other demyelinating diseases have not been clearly identified yet and they are likely to be multiple. However, a majority of studies tend to agree that it is more often due to a failure in oligodendrocyte differentiation than to a lack of available NG2 cells (Franklin and Ffrench-Constant 2008). While demyelinating diseases such as MS could profit from pharmacological treatment promoting NG2 cell proliferation, migration, and differentiation, many congenital myelin diseases are due to genetic mutation that constitutively impairs the ability of NG2 cell to differentiate or to synthetize myelin (Inoue et al. 1999; Touraine et al. 2000). For example, the leukodystrophy Pelizaeus–Merzbacher disease results from mutations in the gene encoding for PLP, a major component of myelin (Mimault et al. 1999). In such conditions, endogenous NG2 cells are unable to form myelin and cell replacement therapies may have to be considered (Goldman et al. 2012).

The myelogenic potential of grafted NG2 cells was first reported in transplanted shiverer mice (Lachapelle et al. 1983), a model of lethal dysmyelination where oligodendrocytes are unable to express MBP and to develop compact myelin (Roach et al. 1985). The shiverer mouse model has been extensively used to demonstrate the myelogenic potential of transplanted precursors from various sources including mouse and human OPCs, immortalized neural stem cells, and primary culture of embryonic neural precursors as well as adult human fetal and adult OPC (Goldman et al. 2012). Recently, using induced human stem cells (hIPSCs), Wang and collaborators were able to generate large quantities of highly enriched OPCs to obtain

whole neuraxis myelination as well as a significant improvement in survival rate of shiverer mice grafted around birth (Wang et al. 2013). However, cell replacement therapies appear more difficult to implement in adults. Indeed, in the adult brain, grafted NG2 cells often exhibit a limited ability to colonize the recipient brain (Blakemore and Irvine 2008; Goldman et al. 2012). This limitation can be overcome by depleting the recipient brain of endogenous NG2 cells (Blakemore and Irvine 2008), but such extensive depletion necessitates aggressive strategies such as extensive irradiation or chemotherapy.

7.3 Neurogenic and Gliogenic Potential of NG2 Cells in Health and Disease

NG2 cells have long been thought to be restricted to an oligodendroglial fate. The first study to suggest otherwise and show that these progenitors could act in vitro as latent neural stem cells was done by Kondo and Raff using purified NG2 cells from rat optic nerve (Kondo and Raff 2000). By treating NG2 cells with fetal calf serum (FCS) or BMP followed by FGF, they were able to generate neurospheres containing neural stem cells that could give rise not only to oligodendrocytes but also to astrocytes and neurons. While this gliogenic and neurogenic potential of NG2 cells has been confirmed by other in vitro studies and is reported in some in vivo models, it appears limited in normal physiological conditions.

7.3.1 Gliogenic Potential of NG2 Cells

A number of studies have reported that NG2 cells can generate astrocytes in several areas of the postnatal brain and spinal cord (Zhu et al. 2008a, b; Guo et al. 2010) as well as more specialized glial cell types such as Bergmann glia in the cerebellum (Chung et al. 2013). However, these astrocytes are likely to be generated during early development since studies tracing specifically the fate of adult cells could not find any astrocytes derived from NG2 cells (Dimou et al. 2008; Rivers et al. 2008; Kang et al. 2010). Therefore, the ability of NG2 cells to generate astrocytes in normal conditions appears limited.

Several studies suggest that NG2 cells could regenerate astrocytes in a pathological context. Indeed, severe injuries such as trauma, hypoxia, and stroke induce proliferation in reactive astrocytes participating to the glial scar (Robel et al. 2011), and there is evidence that a number of reactive astrocytes could be derived from NG2 cells (Tatsumi et al. 2008; Sellers et al. 2009), rather than from protoplasmic astrocytes. However, lineage-tracing studies could not confirm the generation of astrocytes from NG2 cells after brain injury (Dimou et al. 2008). It has also been proposed that NG2 cells can regenerate astrocytes in degenerative diseases such as ALS

(Magnus et al. 2008). Indeed, astrocytes degenerate in the spinal cord of a mouse model of ALS (G93A) (Rossi et al. 2008), and it has been shown that some of the remaining astrocytes incorporate BrdU (Magnus et al. 2008), suggesting that they had been newly regenerated from a proliferative progenitor. However, a recent study analyzing the fate of NG2 cells in the G93A ALS mouse model found that, while NG2 cells regenerate a significant number of oligodendrocytes degenerating in ALS, they do not generate astrocytes over the course of the disease (Kang et al. 2010).

7.3.2 Neurogenic Potential of NG2 Cells

Several studies suggest that neurogenesis from NG2 cells could occur in the hippocampus (Belachew et al. 2003; Aguirre et al. 2004), the olfactory bulb (Aguirre and Gallo 2004), and the cortex (Dayer et al. 2005). It has been reported that NG2 cells purified from the postnatal mouse SVZ can generate GABAergic interneurons, both in vitro (Belachew et al. 2003) and after brain transplantation (Shihabuddin et al. 2000; Aguirre et al. 2004). These studies suggested that the neurogenic potential of NG2 cells is biased toward a GABAergic phenotype, a tendency that could be explained by the common embryonic origin of NG2 cells and GABAergic interneurons in the telencephalon and the adult SVZ. However, experiments using genetic fate-mapping approaches to assess the fate of NG2 cells in vivo have brought contradictory results. Two studies, one using a PLP-CreER transgenic mouse and the other a PDGFR-CreER mouse, reported that NG2 cells generated glutamatergic pyramidal neurons in the piriform cortex, but they did not report any GABAergic neurons generated in the hippocampus (Rivers et al. 2008; Guo et al. 2010). By contrast, two other fate-mapping studies did not observe any neurogenic potential at all for endogenous NG2 cells (Zhu et al. 2008a, b; Kang et al. 2010). These studies used an NG2-Cre transgene (Zhu et al. 2008a, b) and a PDGFR-CreER transgene (Kang et al. 2010) that were both generated through the bacterial artificial chromosome (BAC) modification technique, a method that is believed to allow for transgene expression pattern that is closer to the native gene (Van Keuren et al. 2009).

In conclusion, the ability of endogenous NG2 cells to generate astrocytes and neurons appears limited in vivo, even in pathological conditions. On the other hand, the fact that NG2 cells can be induced in vitro to generate astrocytes and neurons shows that these cells have a latent gliogenic and neurogenic potential that could potentially be recruited through pharmacological or genetic manipulations.

7.4 Conclusion

It has now been clearly established that the unique ability of NG2 cells to proliferate, migrate, and generate new oligodendrocytes is largely responsible for the capacity of the adult CNS to extensively regenerate oligodendrocytes and myelin.

Accordingly, a better understanding on how NG2 cells proceed to regenerate, recolonize, and remyelinate a lesion will bring us closer to develop and/or improve treatments for demyelinating injuries. We have also seen that NG2 cells can be recruited to regenerate neurons and astrocytes, although it appears to necessitate extensive in vitro manipulations, which currently limit their usefulness in a therapeutic context. However, the discovery that NG2 cells can act as neural stem cells in vitro is still recent, and we can remain hopeful that future studies could reveal ways to unleash the stem cell potential of endogenous NG2 cells for therapeutic purposes. Finally, the ability of NG2 cell to recolonize large areas of the brain after depletion provides a new and potentially powerful vector for genetic correction in the CNS. Indeed, it has already been shown that the neonatal graft of healthy NG2 cells can successfully compete with deficient endogenous NG2 cells in order to correct congenital demyelinating disorders. In the adult, whole brain NG2 cell replacement could also be obtained, for example, by combining X-ray irradiation or chemotherapy with autologous or allogeneic NG2 cell transplantation, a strategy that is already used successfully in bone marrow transplantation procedures.

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Chapter 8
Oligodendrocyte Progenitors and Brain Remodeling Following Blood–Brain Barrier Rupture

Praveen Ballabh

Abbreviations

BBB	Blood–brain barrier
BMP	Bone morphogenetic protein
ChABC	Chondroitinase ABC
CNPase	2′,3′-Cyclic nucleotide 3′-phosphodiesterase
COX-2	Cyclooxygenase-2
CSPG	Chondroitin sulfate proteoglycan
GFAP	Glial fibrillary acidic protein
GMH	Germinal matrix hemorrhage
IL1	Interleukin-1
IVH	Intraventricular hemorrhage
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
Ngn	Neurogenin
PDGFRα	Platelet-derived growth factor α
PLP	Proteolipid protein
PVL	Periventricular leukomalacia
Shh	Sonic hedgehog
SVZ	Subventricular zone
TJ	Tight junction
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor

P. Ballabh, M.D. (✉)
Department of Pediatrics, Cell Biology, and Anatomy,
Regional Neonatal Center, Maria Fareri Children's Hospital
at Westchester Medical Center-New York Medical College, Valhalla, NY, USA
e-mail: Pballabh@msn.com

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3_8,

8.1 Introduction

Blood vessels in the brain are unique because they form a blood–brain barrier (BBB). The BBB is a diffusion barrier, which restricts influx of most compounds from blood to brain. Three cellular components of the blood–brain barrier are endothelial cells, astrocyte end feet, and pericytes (Ballabh et al. 2004). Tight junctions (TJ), located between the cerebral endothelial cells, form a diffusion barrier that selectively excludes most blood-borne substances from penetrating the brain (Daneman 2012). Astrocyte end feet tightly ensheath the vessel wall and appear to be critical for induction and maintenance of the TJ barrier. Rupture of the BBB results in brain hemorrhage. In premature newborns, hemorrhage typically occurs in the periventricular germinal matrix. The germinal matrix is located on the head of the caudate nucleus and underneath the ventricular ependyma and consists of a highly vascular collection of glial and neuronal precursor cells (Fig. 8.1). This brain region is selectively vulnerable to hemorrhage and usually bleeds in the first 72 h of life (Ballabh 2010). A substantial bleed in the germinal matrix breaks the underlying ependyma, filling the ventricle with blood. Hence, IVH in premature infants is typically a progression of GMH.

8.2 Germinal Matrix Hemorrhage Induces Brain Remodeling in Preterm Infants

About 12,000 premature infants develop IVH every year in the USA (Ballabh 2010). The frequency of IVH is inversely related to the birth weight and gestational age of infants. In infants <1,000 g, the incidence of IVH is 50–60 %, and in infants 1,000–1,500 g, the incidence is 10–20 % (Courtney et al. 2002). The pathogenesis of IVH is attributed to the intrinsic fragility of the germinal matrix vasculature and fluctuation in cerebral blood flow. The microvasculature of germinal matrix is fragile because there is an abundance of angiogenic blood vessels in this area unlike other regions of brain. These vessels exhibit a paucity of pericytes, immaturity of basal lamina, and deficiency of glial fibrillary acidic protein (GFAP) in the ensheathing astrocyte end feet, which together contribute to the mechanical weakness of the vasculature (Ballabh et al. 2005; El-Khoury et al. 2006; Braun et al. 2007; Xu et al. 2008). The germinal zone is enriched with glial and neuronal progenitor cells, which are proliferating, metabolically active, and consuming large amounts of oxygen. This appears to induce a relative hypoxia, which stimulates production of VEGF and angiopoietin-2. Indeed, the germinal matrix displays rapid endothelial turnover in contrast to neocortical mantle and embryonic white matter.

Hemorrhage in the germinal matrix leads to catastrophic brain injury, damaging the neuronal and glial progenitors and causing extensive remodeling of the

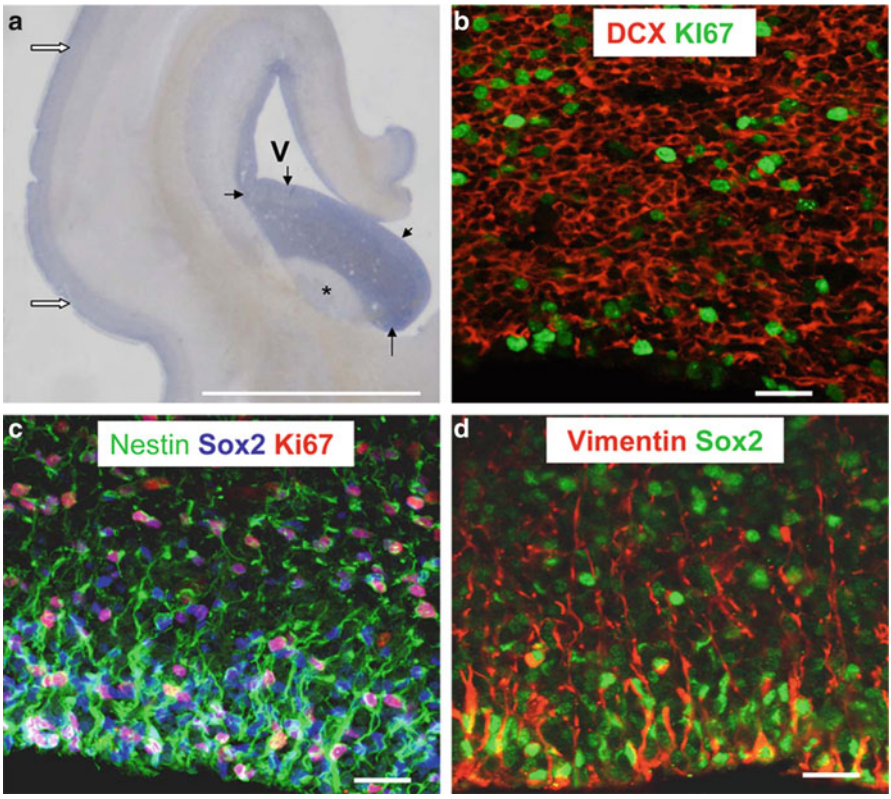

Fig. 8.1 (**a**) Morphology of the germinal matrix. Representative cresyl violet staining of coronal section of the left-sided cerebral hemisphere of a 20-gestational week (gw) fetus. Note: the cortical plate (*white arrow*), germinal matrix (*black arrow*), caudate nucleus (*asterisk*), and lateral ventricle (indicated by "v"). The germinal matrix (violet staining) surrounds the entire ventricle and is most prominent on the head of caudate nucleus. Scale bar, 0.5 cm. (**b**) Representative immunofluorescence of cryosections from 19 gw premature infant double labeled with DCX (migrating neuronal progenitor) and Ki67 (proliferation marker) antibodies in the germinal matrix. Scale bar, 25 μm. (**c**) Triple immunolabeling of coronal sections from 18 gw preterm infant with Sox2 (radial glia), nestin (radial glia and progenitors), and Ki67-specific antibodies. Nestin-positive radial glial processes surround the $Sox2^+$ and $Ki67^+$ nuclear signals in the germinal matrix. Scale bar, 25 μm. (**d**) Double immunolabeling at 20 gw with Sox2 and vimentin antibodies. Note the predominance of Sox2+ cells in the germinal matrix which are embedded into vimentin. Scale bar, 25 μm

periventricular white matter. This results in impaired myelination and alteration of cerebral cortical neurons. In addition, there is an occlusion of cerebral spinal fluid circulation. Consequently, the surviving infants exhibit a number of neurologic sequelae, including cerebral palsy, reduced cortical growth, mental retardation, hydrocephalus, and behavioral disorders. Greater severity of IVH leads to a worse neurological outcome.

8.3 Oligodendrocyte Specification and Maturation During Normal Brain Development

Oligodendrocytes progressively develop as a well-defined lineage, which is characterized by the expression of stage-specific antigens. Mature forms exhibit more complex morphology relative to less mature forms. The four successive stages of oligodendrocytes include early oligodendrocyte progenitors, late oligodendrocyte progenitors (pre-oligodendrocyte), immature oligodendrocytes, and mature oligodendrocytes (Fig. 8.2) (Back et al. 2001). Early oligodendrocyte progenitors are identified by reactivity to PDGFRα or NG2 antibodies. Late oligodendrocyte progenitors (pre-oligodendrocytes) are multipolar, mitotically active, and reactive to O4 antibody (Fig. 8.3). Immature oligodendrocytes are postmitotic, complex multipolar cells that express galactocerebroside (O1 antigen) and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase). Mature oligodendrocytes synthesize the major myelin proteins—myelin basic protein (MBP) and proteolipid protein (PLP) (Pfeiffer et al. 1993). Oligodendrocytes in the white matter and cerebral cortex originate from the lateral and medial ganglionic eminence and cortical subventricular zone (Rakic and Zecevic 2003). During human pregnancy, 23–35 weeks of gestation is the window when the oligodendrocyte lineage progresses through these phenotypic stages. While pre-oligodendrocytes are vulnerable to oxidative stress and to hypoxic-ischemic insults, immature oligodendrocytes are relatively resistant to these events (Back et al. 2002).

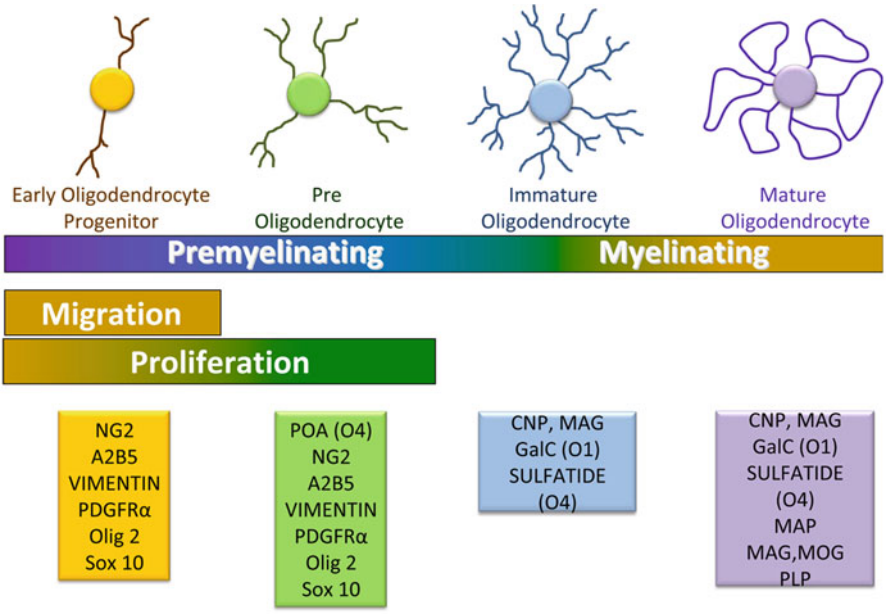

Fig. 8.2 Specification and maturation of oligodendrocytes into four successive stages as depicted in the image. Antigenic markers used to identify each of these stages are also shown

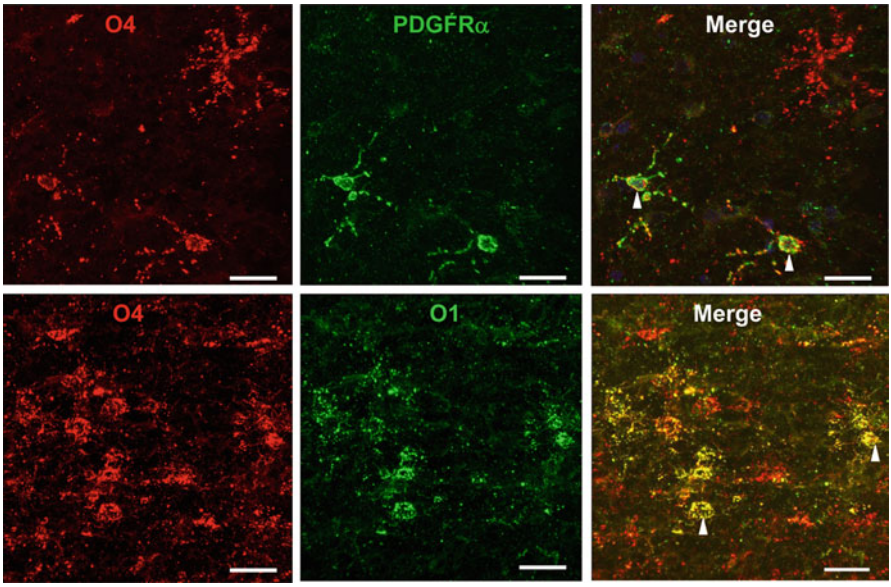

Fig. 8.3 Maturation of oligodendrocytes. *Upper panel*: Representative double immunolabeling of the cryosections from the forebrain of 23 gw preterm infants using a combination of O4 and PDGFRα. Note some PDGFRα$^+$ progenitors also express O4$^+$ antigen (*upper panel*, *arrowhead*). *Lower panel*: Cryosections were double labeled with O4 and O1 antibody. Note cells expressing both O4 and O1 antigen are myelinating (immature, *arrowhead*) oligodendrocytes. Scale bar, 25 μm

8.3.1 Specification of Oligodendrocytes

As oligodendrocyte maturation progresses from specification to terminal differentiation, a number of transcription factors play essential roles in this process. These transcription factors can be classified as basic–helix–loop–helix (excitatory bHLH: Olig1, Olig2, Mash1; inhibitory bHLH: Id2, Id4), homeodomain (Nkx 2.2, Nkx 6.1), and high mobility group (Sox 9, Sox 10, and Sox17), which are the key regulators of oligodendrogenesis (Wegner 2008). Intriguingly, sonic hedgehog (Shh), bone morphogenetic protein (BMP), notch, and Wnt signaling pathways control these transcription factors (Fig. 8.4).

Olig2 is critical for specification of oligodendrocytes because its deficiency limits the PDGFRα expression to focal areas in the ventral forebrain (Nicolay et al. 2007). However, double knock out of Olig1 and Olig2 genes results in a total loss of PDGFRα expression (Zhou and Anderson 2002). This implies that both Olig1 and Olig2 are essential for oligodendrocyte specification in the forebrain; however, Olig2 plays the major role in this process. Nkx6.1 and Gli2 mutant mice also display reduced Olig2 expression with a concomitant decrease in PDGFRα levels (Cai et al. 2005; Vallstedt et al. 2005). Recent studies have revealed that Mash1 is

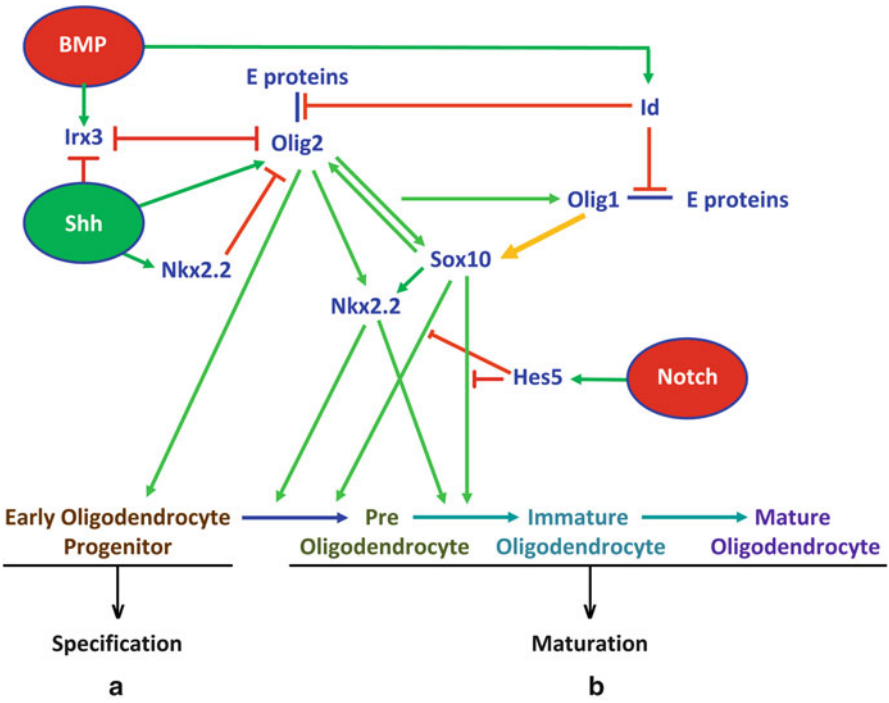

Fig. 8.4 Transcriptional network involved in the specification and maturation of oligodendrocyte. (**a**) Oligodendrocyte specification: Development of early oligodendrocyte progenitors (PDGFRα+) marks the specification of oligodendrogenesis and is regulated by Olig2, Nkx2.2, Pax6, and other transcription factors. These transcription factors are regulated by bone morphogenetic protein, sonic hedgehog, and notch signaling pathways. (**b**) Maturation of oligodendrocytes is also controlled by a number of transcription factors—Olig1, olig2, Nkx2.2, Sox10, and Hes5, which are regulated by bone morphogenetic protein, sonic hedgehog, and notch signaling pathways

essential for specification of a subset of oligodendrocytes originating from the ventral telencephalon (Parras et al. 2007).

Shh is a key inducer of Olig1 and Olig 2 transcription factors (Lu et al. 2000; Alberta et al. 2001). Indeed, Shh knockout mice fail to express Olig1, Olig2, and PDGFRα receptor throughout the brain (Tekki-Kessaris et al. 2001). In contrast to Shh, BMP protein represses Olig2 expression (Mekki-Dauriac et al. 2002). The exact mechanism, though unknown, is speculated to be through indirect enhancement of Irx3 transcription factor expression (Novitch et al. 2001). Importantly, blocking BMP signaling with noggin expands the population of late oligodendrocyte progenitors (Hall and Miller 2004; Sabo et al. 2011).

In the telencephalon, neurogenesis precedes oligogenesis, and the germinal matrix forms neuronal progenitors before forming pre-oligodendrocytes. As the neuronal–glial switch is taking place, a complex set of molecular changes occurs. Loss- and gain-of-function studies in mice suggest that downregulation of proneural activity is critical for the switch from neurogenesis to gliogenesis (Zhou et al. 2001). Downregulation of Ngn1/2 has been identified as the determinant of the

neuronal–glial switch and thus specification of oligodendrocytes (Novitch et al. 2001). The role of Delta–Notch signaling in oligodendrocyte specification has also been deciphered. Notch signaling is essential to maintain a subset of Olig2^{+} neural precursors until gliogenesis is initiated (Rowitch 2004). Notch plays a permissive role, but not instructive, for glial fate acquisition as forced expression of Notch 1 receptor does not affect the timing of oligodendrocyte generation (Givogri et al. 2002).

8.3.2 Maturation of Oligodendrocytes

After specification of oligodendrocytes, these cells undergo successive stages of maturation including early oligodendrocyte progenitors, pre-oligodendrocytes, immature oligodendrocytes, and mature oligodendrocytes. While pre-oligodendrocytes are nonmyelinating oligodendrocytes, immature and mature oligodendrocytes are myelinating.

8.3.2.1 Inhibitors of Maturation

Overall, Notch, BMP, and retinoic acid inhibit maturation of oligodendrocytes during the perinatal period (Nicolay et al. 2007). In addition, hyaluronan in animal models of disseminated sclerosis has been shown to suppress myelination (Emery 2010). Culture experiments have shown that Notch activation arrests maturation of pre-oligodendrocytes into immature (GalC+) oligodendrocytes (Wang et al. 1998). Accordingly, mice deficient in Notch 1 exhibit elevated MBP and PLP (Givogri et al. 2002). Hes5 transcription factor, a downstream molecule of Notch signaling, also inhibits differentiation of oligodendrocyte progenitors and promotes differentiation of astrocytes (Kondo and Raff 2000).

BMP suppresses both specification and maturation of oligodendrocyte progenitors. This morphogen induces Id2 and Id4 transcription factors that inhibit maturation of pre-oligodendrocytes into immature oligodendrocytes in culture experiments (Samanta and Kessler 2004). In fact, Id2 and Id4 are negative regulators of Olig1 and Olig2 and interfere with Olig/E protein heterodimerization (Samanta and Kessler 2004). Hence, BMP arrests oligodendrocyte maturation by elevating Id2 and Id4 expression, which hinders Olig1 and Olig2 function.

8.3.2.2 Activators of Maturation

A number of transcription factors, including Olig1, Olig2, Nkx2.2, Nkx6.2, and MyT1, support maturation of oligodendrocytes (Wegner 2008). While Olig1 plays a minor role in oligodendrocyte specification, it plays a key role in oligodendrocyte maturation (Lu et al. 2002; Xin et al. 2005). This is evident from observations made in Olig1-deficient mice, in which expression of MBP and PLP are markedly

impaired in the corpus callosum of the forebrain (Lu et al. 2002). Similarly, the density of GalC$^+$ and PLP$^+$ oligodendrocytes is significantly reduced in spinal cord culture treated with Olig2 antisense (Fu et al. 2002). Hence, both Olig1 and Olig2 promote maturation of oligodendrocyte progenitors. Nkx2.2 is a transcriptional repressor of myelin genes; however, Nkx2.2 induces PLP gene expression (Qi et al. 2001). Sox10 is another transcription factor that promotes terminal differentiation of oligodendrocytes and induces transcription of a number of myelin genes—MBP, PLP, and MAG (Stolt et al. 2002).

Together, oligodendrogenesis is a dynamic interplay of a number of excitatory and inhibitory transcription factors, which are regulated by Shh, Notch, and BMP signaling pathways.

8.3.3 Origin of Oligodendrocyte Progenitors in the Normal Embryonic Brain

Oligodendrocyte progenitors develop primarily in the ventral regions of the telencephalon (Spassky et al. 1998; Tekki-Kessaris et al. 2001). Olig1 and Olig2 transcription factors and Shh signaling are the key regulators of oligodendrogenesis in these brain regions (Spassky et al. 2001). Olig2 gene expression largely overlaps Mash1, and PDGFRα$^+$ and Sox10$^+$ progenitors co-express Olig1 in the medial ganglionic eminence (Tekki-Kessaris et al. 2001). Together, it appears that oligodendrocyte progenitors develop from a subset of neural cells in the medial ganglionic eminence. In addition, cortical progenitors in the subventricular zone may give rise to oligodendrocytes at later stages of development.

8.4 Remodeling of Periventricular Region in Intraventricular Hemorrhage (IVH): Initiation of Inflammation

The key factors that can contribute to oligodendrocyte injury after IVH are as follows: (a) an inflammatory response resulting in elevation of pro-inflammatory cytokines and reactive oxygen species, (b) oxidative stress secondary to the presence of iron in the hemoglobin, (c) glutamate excitotoxicity induced by thrombin and other blood components, (d) compression and possible ischemia of the periventricular region, (e) destruction of germinal matrix, and (f) increase in intracranial pressure (Vinukonda et al. 2012). Furthermore, the onset of IVH might jeopardize cerebral blood flow and autoregulation in premature infants.

We have evaluated IVH-induced inflammatory response in our preterm rabbit model of IVH (Georgiadis et al. 2008; Hallevi et al. 2011). There is neutrophil and microglia infiltration around the ventricle, which progressively increases in the first

3 days of life. There is activation of caspase-3, caspase-7, and caspase-9 in rabbit pups with IVH, suggesting stimulation of both intrinsic and extrinsic apoptotic pathways (Vinukonda et al. 2010). The TUNEL$^+$ cells are abundantly present around the ventricle, and their densities are highest at day 1, decreasing progressively by day 3 (Georgiadis et al. 2008). Neuronal degeneration also occurs in the germinal matrix following IVH (Georgiadis et al. 2008).

IVH induces upregulation of COX-2 and elevates pro-inflammatory cytokine levels including TNF-α and IL1-β (Vinukonda et al. 2010). The expression of these molecules has been assessed in the first 72 h of life after the induction of hemorrhage. The expression of all three—COX-2, TNF-α, and IL1-β—is elevated in rabbit pups with IVH compared to matched controls without IVH at 24, 48, and 72 h after the onset of hemorrhage (Vinukonda et al. 2010). Importantly, IVH also induces enhanced oxidative–nitrosative stress in the brain regions around the ventricle in these animals, and by extension in preterm human infants (Zia et al. 2009). Free-radical generation could be a function of a number of oxidative pathways, including NADPH oxidase, cyclooxygenase, xanthine oxidase, or nitric oxide synthase and mitochondria (Zia et al. 2009). In preterm rabbits, NADPH oxidase is the major source of free-radical generation (Zia et al. 2009). Together, the onset of IVH initiates a robust inflammatory response resulting in cellular infiltration, apoptosis, cytokine elevation, and oxidative stress.

8.5 Brain Hemorrhage Disrupts Oligodendrocyte Survival and Maturation

8.5.1 Oligodendrocyte Injury in Intraventricular Hemorrhage

IVH in preterm infants is associated with white matter injury, which has been revealed in a number of reports (DI Rushton and Durbin 1985; Armstrong et al. 1987). White matter injury could be either localized or generalized (Back 2006). Localized white matter damage is known as periventricular leukomalacia (PVL). PVL is a cystic lesion in the periventricular white matter outer to the anterior or posterior horn of the lateral ventricles. Diffuse white matter injury is more common than cystic PVL and consists of areas of hypomyelination with preservation of axons (Back 2006). Our recent work has started to unfold the mystery of white matter injury in IVH (Vinukonda et al. 2010, 2012, 2013; Dummula et al. 2011). In our rabbit pup model of IVH, the onset of hemorrhage induces apoptosis of oligodendrocyte progenitors and suppresses proliferation of PDGFRα$^+$ and Olig2$^+$ oligodendrocyte progenitors (Vinukonda et al. 2010; Dummula et al. 2011). In addition, it arrests maturation of oligodendrocytes in the pre-oligodendrocyte stage, resulting in abundance of pre-oligodendrocytes and paucity of immature and mature oligodendrocytes. Mature and immature oligodendrocytes produce myelin, and thus, their scarcity results in hypomyelination.

The bHLH and high mobility group transcription factors have been assessed in preterm rabbits with IVH (Vinukonda et al. 2010; Dummula et al. 2011). The transcription factors enhancing myelination including Olig2 and Sox10 are reduced, while inhibitory transcription factors—Id2 and Id4—are elevated in animals with IVH.

8.5.2 *Intraventricular Hemorrhage Disturbs Signaling Pathways Regulating Oligodendrogenesis*

Little is known about how Notch, Shh, BMP, and Wnt signaling pathways control oligodendrogenesis in premature human infants or in models of intraventricular hemorrhage. However, recent work in both autopsy materials and our rabbit model has revealed that BMP is elevated in IVH (Vinukonda et al. 2010; Dummula et al. 2011). In fact, BMP levels remained high for the first 2 weeks after the initiation of IVH in our rabbit model. Importantly, the key downstream molecules for BMP signaling, phospho-Smad 1/5/8 are also elevated in rabbits with IVH. This confirms that the BMP cascade is active, which can potentially suppress maturation of oligodendrocytes.

Notch, Shh, and Wnt signaling has not been assessed in any animal model of IVH or in autopsy materials from premature infants. However, it is likely that these signaling pathways are affected, which might be contributing to the myelination failure in IVH.

8.5.3 *Remodeling of the Extracellular Matrix in Newborns with IVH*

The neural cells in the brain are embedded in the extracellular matrix, which is composed of proteoglycans, hyaluronan, and several secreted glycoproteins. There is an abundance of hyaluronan, which binds with proteoglycans, tenascins, and link proteins. Chondroitin sulfate proteoglycans (CSPGs) include lecticans, phosphacan, and NG2, and the families of lecticans comprise aggrecan, versican, neurocan, and brevican. CSPGs are upregulated in animal models of cerebral hypoxia—ischemia, surgically injured cerebral cortex and spinal cord, and inflammatory brain disease. CSPGs limit plasticity of the brain after injury, and breakdown of CSPGs by chondroitinase ABC (ChABC) treatment promotes plasticity of the brain structures. Elevated CSPGs in the ECM reduce migration and formation of process outgrowth of oligodendrocyte progenitors and impede movement of extracellular fluid in the brain (Sykova et al. 2001; Siebert and Osterhout 2011). Similar to other brain injuries, there is elevation in the level of neurocan, brevican, aggrecan, phosphacan, and versican, whereas NG2 expression is reduced in premature rabbit pups and

human infants with IVH compared to controls without IVH (Vinukonda et al. 2013). The expression of other components of the extracellular matrix—hyaluronan, tenascins, and others—has not been evaluated in any animal model of IVH or human infants with IVH.

8.6 Neuroprotection in Infants with Intraventricular Hemorrhage

A number of strategies can be employed to protect oligodendrocytes and offset the brain injury caused by IVH. The onset of IVH damages the periventricular white matter by production of inflammatory mediators, glutamate excitotoxicity, and oxidative stress. Therefore, suppressing inflammation, glutamate excitotoxicity, or free-radical generation is likely to reduce brain injury in these infants. In addition, modulating signaling pathways controlling oligodendrogenesis, including BMP, Shh, Notch, and Wnt, might enhance oligodendrocyte maturation and restore myelination in the survivors of IVH. Stem cell therapy for replacement of oligodendrocyte and fostering regeneration are other important strategies that are yet to be investigated.

8.6.1 Cox-2 Inhibition to Suppress Inflammation

COX-2—an inducible enzyme catalyzing synthesis of prostanoid—plays a key role in cerebral pathologies associated with inflammation, oxidative injury, and glutamate excitotoxicity (FitzGerald 2003; Minghetti 2007); (Candelario-Jalil and Fiebich 2008). COX-2 activation increases PGE2 levels, which stimulates G-protein-coupled receptors, including prostanoid receptors EP1, EP2, EP3, and EP4. COX-2 is mechanistically linked to TNF-α (Vinukonda et al. 2010). Studies have shown that COX-2, EP1, and TNF-α are upregulated in IVH, and inhibition of each of the three molecules offers short-term neuroprotection in separate preclinical testing. In addition, COX-2 inhibition by celecoxib alleviates neurological impairment, improves myelination, reduces gliosis, and induces neurological recovery in adult animals after intracerebral hemorrhage (Chu et al. 2004). Selective COX-2 inhibition also alleviates motor impairment in the G93A superoxide dismutase mouse model of amyotrophic lateral sclerosis (Pompl et al. 2003). Hence, COX-2 inhibition might hold promise for neuroprotection in human infants with IVH.

8.6.2 Reducing Oxidative Stress

IVH results in excessive free-radical generation, which releases excitatory amino acids, thereby damaging neurons, neurofilaments, and glial cells. In our rabbit

model of IVH, activation of NADPH oxidase is the predominant mechanism of free-radical generation (Zia et al. 2009). Accordingly, apocynin, an NADPH inhibitor, suppresses reactive oxygen species production, reduces cell death, and confers neuroprotection (Zia et al. 2009). However, inhibitors of xanthine oxidase (allopurinol), COX-2 (indomethacin), or nitric oxide synthases do not reduce oxidative stress or cell death in rabbit pups with IVH (Zia et al. 2009). Further studies are needed to determine long-term neuroprotective effects of apocynin and other more promising agents, which can be tested in clinical trials and can successfully emerge as a therapeutic agent for premature infants with hemorrhage.

8.6.3 BMP Inhibition

BMP overexpression reroutes oligodendrocyte progenitors to an astrocyte lineage, enhances astrocytosis, and suppresses oligodendrocyte differentiation by downregulating Olig1 and Olig2 transcription factors (Nicolay et al. 2007). IVH results in an increase in BMP-4 protein in both rabbits and human infants and is associated with maturational failure of oligodendrocyte progenitors and gliosis (Vinukonda et al. 2010; Dummula et al. 2011). These increases are seen predominantly in the periventricular germinal matrix and white matter. Accordingly, there is an elevation in levels of phospho-Smad1/5/8 in rabbit pups with IVH indicating enhanced bone morphogenetic protein signaling in pups with IVH (Dummula et al. 2011). More importantly, BMP inhibitor—noggin—restores myelination, reduces gliosis, and increases neurological recovery in rabbit pups with IVH. Noggin is an extracellular BMP antagonist that binds with BMP2/4, thus blocking its binding to receptors. BMP4 treatment on cerebral oligodendrocyte precursor cells decreases MBP and PLP expression via activation of Id-2 and Id-4 and inhibition of Olig1 and Olig2 genes (Yanagisawa et al. 2001; Cheng et al. 2007), and conversely, overexpression of BMP antagonist—noggin—in transgenic mice reduces ischemic brain injury by increasing the density of oligodendrocyte progenitors (Samanta et al. 2009). Furthermore, chordin, a BMP antagonist, enhances oligodendrogenesis in the SVZ cells in both in vivo and in vitro experiments (Jablonska et al. 2010). Hence, BMP inhibition can be an important strategy that can potentially be translated into patient treatment. A major obstacle to this approach is the requirement of intracerebral rather than systemic administration of the medication to downregulate BMP4 in the brain. However, ventricular drainage using frontal and occipital catheters, irrigation with artificial CSF, and fibrinolytic therapy have been tried in a randomized clinical trial in human premature infants without success (Whitelaw et al. 2007). Thus, intracerebral administration of BMP inhibitors in the cerebral ventricle of premature infants, even though invasive and involving risk, is possible. Unfortunately, a systemic use of small molecule BMP inhibitor that penetrates the blood–brain barrier has not undergone clinical trial for any disease (Hong and Yu 2009).

8.6.4 Inhibition of Wnt Signaling

Wnt signaling drives proliferation and inhibits terminal differentiation of oligodendrocyte progenitors into mature oligodendrocyte both during development and remyelination, and inhibition of Wnt signaling induces robust increases in the production of oligodendrocyte progenitors (Fancy et al. 2009; White et al. 2010). Therefore, it is tempting to speculate that inhibition of Wnt signaling might restore oligodendrogenesis and myelination in infants with IVH. A number of inhibitors of the Wnt signaling pathway, including imatinib mesylate, lithium, curcumin, NSAIDs, and flavonoid, are available, which can be potentially used for these experiments.

8.6.5 Degradation of CSPGs

The development of IVH elevates CSPG—neurocan, brevican, versican, aggrecan, and phosphacan levels—and reduces NG2 expression in rabbit pups and infants with IVH (Vinukonda et al. 2013). However, degradation of CSPGs by in vivo ChABC treatment failed to promote differentiation of OL, myelination, and clinical recovery in the surviving pups (Vinukonda et al. 2013). Moreover, ChABC treatment does not alleviate gliosis and ventriculomegaly in these pups. The study refutes the speculation of several in vitro and in vivo studies that ChABC treatment might enhance myelination and clinical recovery after brain injuries.

8.7 Conclusion

IVH–GMH is typically a rupture of the blood–brain barrier in premature infants, which leads to remodeling of the periventricular germinal matrix and hypomyelination of the white matter. The injury to the white matter is caused by iron, thrombin, complement, and other components of the blood, along with the adverse hemodynamic changes in the periventricular cerebral vasculature. Induction of inflammation, production of pro-inflammatory cytokines, infiltration of inflammatory cells, generation of free radicals, and glutamate excitotoxicity are the key events that initiate the injury. These factors contribute to degeneration and arrested maturation of oligodendrocytes and trigger astrocytosis. The molecular pathways underlying the morphological remodeling include Shh, Notch, and BMP, which control oligodendrogenesis through a number of transcription factors. Modulating the transcription factors through these signaling pathways might restore oligodendrocyte maturation and neurological function in preterm infant with IVH.

Acknowledgements Authors thank Joanne Abrahams for the assistance with images.

Source of funding: NIH/NINDS grant RO1 NS071263 (PB).

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Chapter 9
Genomic Integrity of Embryonic and Neural Stem Cells

Nathalie Lefort and Marc Peschanski

9.1 Introduction

9.1.1 Pluripotent Stem Cells

Totipotent and pluripotent stem cells (PSCs) are the only cells able to generate all tissues of an organism. The first PSC lines were isolated nearly 30 years ago from mouse embryos (Evans and Kaufman 1981; Martin 1981). It was only 17 years later that the first human PSCs have been successfully derived from the inner cell mass of the blastocyst (5.5–7.5 days post-fertilization) (Thomson et al. 1998). Embryonic stem cell (ESC) lines have the ability to divide virtually endlessly while maintaining the capacity, under specific conditions, to differentiate into all cell types of the organism. A new type of PSCs, "induced PSCs" (iPSCs), sharing common properties with ESCs (self renewal, pluripotency), have become accessible to the scientific community. In 2006 Yamanaka's group produced iPSCs from the mouse (Takahashi and Yamanaka 2006) and then in 2007 from human cells (Takahashi et al. 2007; Yu et al. 2007). This discovery earned him a Nobel Prize in 2012. IPSCs, originating from diverse somatic cells including fibroblasts (skin, lung, foreskin) (Takahashi et al. 2007; Yu et al. 2007), keratinocytes (Aasen et al. 2008), melanocytes (Utikal et al. 2009), blood cells (Loh et al. 2009; Okita et al. 2013) and adipocyte tissue (Esteban et al. 2010), are obtained from reprogramming by ectopic expression of defined transcription factors. The reprogramming approaches rely on the delivery of the pluripotent factors via integrating vectors (Takahashi et al. 2007; Yu et al. 2007),

N. Lefort (✉) • M. Peschanski (✉)
French Institute of Health and Medical Research, UMR 861, I-Stem, French Muscular Dystrophy Association, Institute for Stem Cell Therapy and Exploration of Monogenic Diseases, University of Évry Val d'Essonne, Evry cedex, France
e-mail: nlefort@istem.fr; mpeschanski@istem.fr

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3_9,

via excisable integrating vectors (Soldner et al. 2009; Woltjen et al. 2009; Kaji et al. 2009) or via nonintegrative vectors such as adenovirus (Stadtfeld et al. 2008) or Sendaï virus (Fusaki et al. 2009; Ban et al. 2011). The delivery can also be transgene-free [RNA (Yakubov et al. 2010) or protein (Zhou et al. 2009; Kim et al. 2009)].

Therefore, human ESCs and iPSCs represent great promise to provide an unlimited alternative source for regenerative medicine. IPSC lines have the added advantage of opening the possibility of selecting human leukocyte antigen (HLA) matching donors (Gourraud et al. 2012).

9.1.2 Pluripotent Stem Cells in Regenerative Medicine

Neural derivatives (central or retinal nervous system) of human ESCs have been or are currently being used for cell therapies in four clinical FDA-approved trials. The first one has been run since October 2010 by Geron Corporation (NCT01217008) to test the safety of hESC-derived oligodendrocytes in patients with spinal cord injury. This trial was stopped, however, due to financial issues at the company. The second and third hESC trials were launched in November 2010 by Advanced Cell Technology (ACT) to test the safety of hESC-derived retinal pigmented epithelial cell (RPE) therapy for Stargardt's macular dystrophy (SMD) (USA trial: NCT01345006; UK trial: NCT01469832; Korea trial: NCT01625559) and for dry age-related macular degeneration (USA trial: NCT01344993; Korea trial: NCT01674829). A fourth trial conducted by Pfizer (NCT01691261) started in September 2013 and aims to investigate the safety of using transplanted retinal cells derived from hESCs to treat patients with advanced Stargardt disease.

9.1.3 One Dark Side of Pluripotent Stem Cells and Their Progenies: Genomic Instability

Human PSCs and their progeny offer a limitless cell source for regenerative medicine. However, their extensive culture carries the risk of generating genomic abnormalities. Such a genetic instability has already been observed (Lefort et al. 2009; Lund et al. 2012a; Martins-Taylor and Xu 2012; Nguyen et al. 2013). The identification of markers allowing the sorting of karyotypically abnormal cells should be an easy way to ensure that one works with cells free of chromosomal defects. However, such biomarkers of instability have remained elusive. The CD30 marker has been considered since its expression was associated with aneuploidies (Herszfeld et al. 2006). However, it was later shown that its expression was not restricted to karyotypically abnormal cells (Lagarkova et al. 2008; Thomson et al. 2008; Harrison et al. 2009; Mateizel et al. 2009), but rather induced by ascorbic acid in the media (Chung et al. 2010). Currently the only way to study chromosomal integrity is, therefore, to stop a cell culture and karyotype it.

In this review, we will focus first on the main techniques used to explore genomic integrity, before discussing the current state of knowledge on the changes registered in pluripotent stem cells and their progenies.

9.2 Methods to Control Genomic Integrity

9.2.1 Metaphase-Based Analysis

9.2.1.1 Chromosomal Banding

The first banding methods (e.g. Q, G or R bands) were developed in the early 1970s (Caspersson et al. 1970; Seabright 1971; Dutrillaux et al. 1972). They are based on the staining of chromosome segments, with a dye (quinacrine for Q bands, Giemsa for G and R bands). Bands are clearly visible after chromosome condensation and allow detection of 400–600 bands resulting in an average resolution of around 5–10 megabases depending on the region in the genome. They are classified according to the technique used to produce them. G (Giemsa)-banding relies on trypsin denaturation of chromosomes, whereas R (reverse)-banding method is based on their thermal denaturation. In G-banding, the dark regions tend to be AT-rich, while they are GC-rich with R-banding. This technique allows the identification of abnormalities of chromosome number and structure (Fig. 9.1). The resolution of this method is therefore poor and depends on how condensed the chromosomes are, but its sensitivity is quite high since single cells are examined. The quality of the interpretation relies on the skill of a cytogeneticist. G-banding is considered to be the gold standard in pluripotent stem cell laboratories and is strongly advised as a regular control for genomic stability (ISCI 2009). The first studies reporting genomic instability in pluripotent stem cells used this technology (Draper et al. 2004; Inzunza et al. 2004; Brimble et al. 2004). These results have now been confirmed by many groups.

9.2.1.2 Whole Chromosome Painting

Twenty-five years after the introduction of conventional banding methods, Schrock (Schrock et al. 1996) introduced a new karyotyping technology based on fluorescent in situ hybridization (FISH). The two variants of this approach are spectral karyotyping (SKY) and multicolor FISH (mFISH). Each of the 24 different human chromosomes is stained with chromosome-specific probes labeled with different fluorochrome combinations. Each chromosome pair has then a specific colour, making interpretation easier, but this technique is also somewhat expensive. This method's sensitivity is as fine as traditional karyotyping with a resolution of around five megabases, depending on the chromosomal location (Fig. 9.2). Abnormalities of chromosome number and interchromosomal rearrangements can be detected.

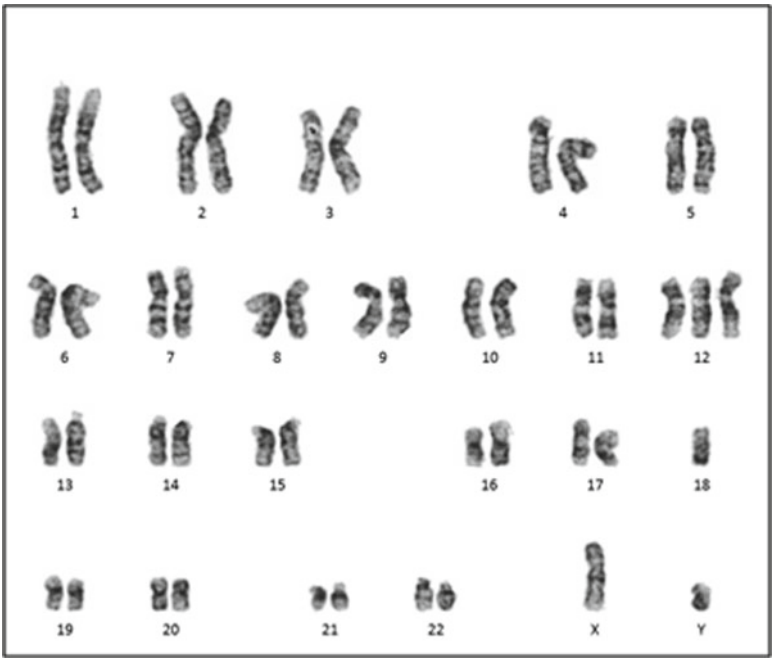

Fig. 9.1 Representative picture of G-banded karyotype of a trisomic 12 male cell line (47, XY, +12)

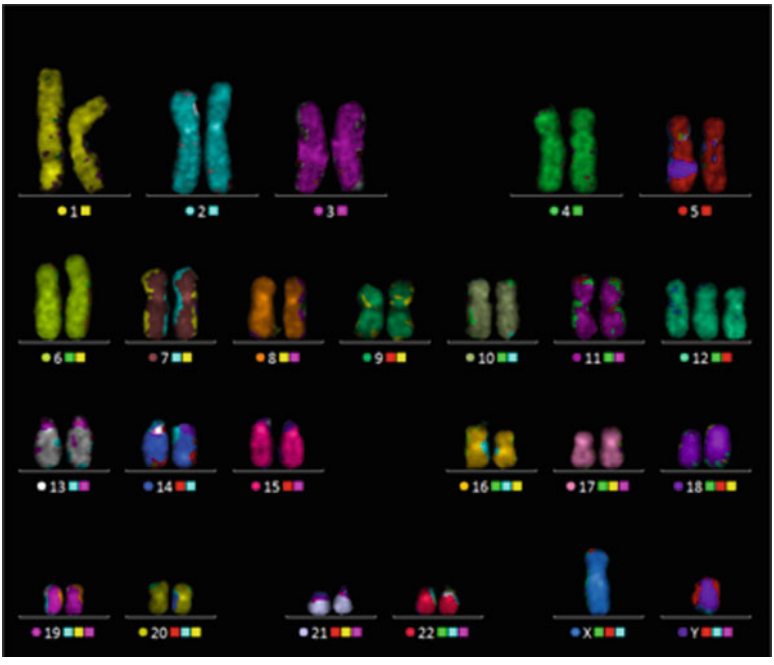

Fig. 9.2 Representative picture of multicolour fluorescent karyotype of a male cell line with karyotype 47, XY, +12

In contrast, intrachromosomal defects such as inversion, deletion and duplication are missed since these are shown with the same colour. Whole chromosomal painting has been successfully applied to cultures of human pluripotent stem cells and led to the identification of chromosomal abnormalities (Imreh et al. 2006; Catalina et al. 2008).

9.2.1.3 Fluorescent In Situ Hybridization

FISH is used to pinpoint aneuploidies or to detect deletions and duplications of suspected regions of interest that are beyond the resolution of conventional karyotyping. However, a prior knowledge of the genomic region of interest is required. In addition, small deletions may not be detected by FISH, since the abnormalities need to be larger than the probe used. In a few studies, FISH has been applied to screen hundreds of cells looking for aneuploidies. However, the choice of the probes was frequently inappropriate since probes directed against chromosomes 13, 18, 21 and X were most often used. These aneuploidies are the most commonly seen in prenatal diagnosis but are not common in PSC culture, except for chromosome X.

9.2.2 Genomic DNA-/RNA-Based Analysis

9.2.2.1 Bead-Bound Bacterial Artificial Chromosome Probes

Bead-bound bacterial artificial chromosome (BAC) technology aims to evaluate DNA copy number variations at the chromosome arm resolution using BAC probes immobilized onto colour-encoded beads distinguishable by a fluorometer. Telomeric and centromeric regions of each chromosome will thus be recognized in a single assay. This rapid, high-throughput and cost-efficient method allows for the detection of arm-specific aneuploidies (Fig. 9.3). The resolution is then really low compared to all the other tools. This assay does not detect balanced rearrangement or low level mosaicism (30 %). Since these technologies do not require cells to be in metaphase, abnormal cells with a lower mitotic rate can be revealed. This method recently enabled high-throughput karyotyping of human pluripotent stem cells (Lund et al. 2012b).

9.2.2.2 Array-Based DNA Analyses

Advances in molecular cytogenetics have improved the resolution of testing through the application of array-based molecular analyses (also called virtual karyotypes). Molecular karyotyping such as array-based comparative genomic hybridization (aCGH) or single nucleotide polymorphism (SNP) array has a much higher resolution, ranging from less than 1 Mb to less than 100 kb depending on the probe density.

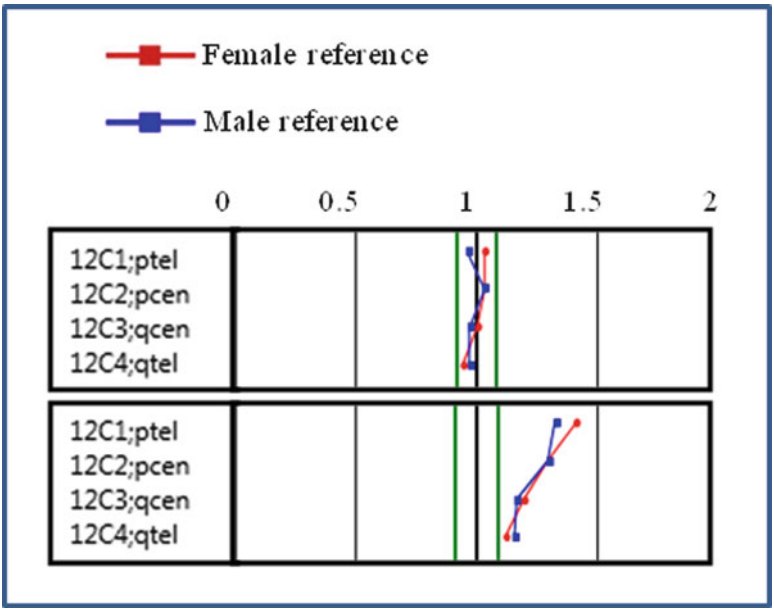

Fig. 9.3 Chromosome 12 analysis by BASs-on Beads method. The *red* and *blue lines* indicate chromosomal signal ratios against female (*red*) and male (*blue*) reference DNA with normal karyotypes. Each square corresponds to a bead. The *green lines* indicate the upper and lower thresholds outside which amplification and deletion are detected. For normal chromosomes 12, both signals calculated against female and male references reside inside the reference area around value 1 (*upper panel*). In contrast for abnormal karyotype, both signals are located outside the reference area (*lower panel*)

They have provided detection of subchromosomal defects in PSCs, such as copy number variations (Maitra et al. 2005; Josephson et al. 2006) (Fig. 9.4). SNP arrays offer several advantages over aCGH: they can detect copy-neutral loss of heterozygosity (cnLOH) events also named acquired uniparental disomy (UPD). In addition, SNP array can detect polyploidy in the only cases where cells possess an odd number of haploid sets (i.e. triploidy, pentaploidy) which cannot be detected in aCGH because test and control samples contain the same proportion of haploid sets per hybridized DNA. In the case of tetraploidy (more specifically autotetraploidy) all DNA-based methods failed to detect the abnormality because diploid or autotetraploid cells will display the same profile. As with any other DNA-based analyses, these methods do not require cell culture and have the advantage of not only considering the few dividing cells but also the whole cell population. Several studies on dozens of pluripotent stem cell lines have highlighted the high prevalence of these subchromosomal genomic alterations (Narva et al. 2010; Amps et al. 2011; Taapken et al. 2011). These two techniques have drawbacks, as compared to metaphase-based methods: they are not able to detect balanced rearrangements or low level mosaicism (20 %), and they are quite expensive.

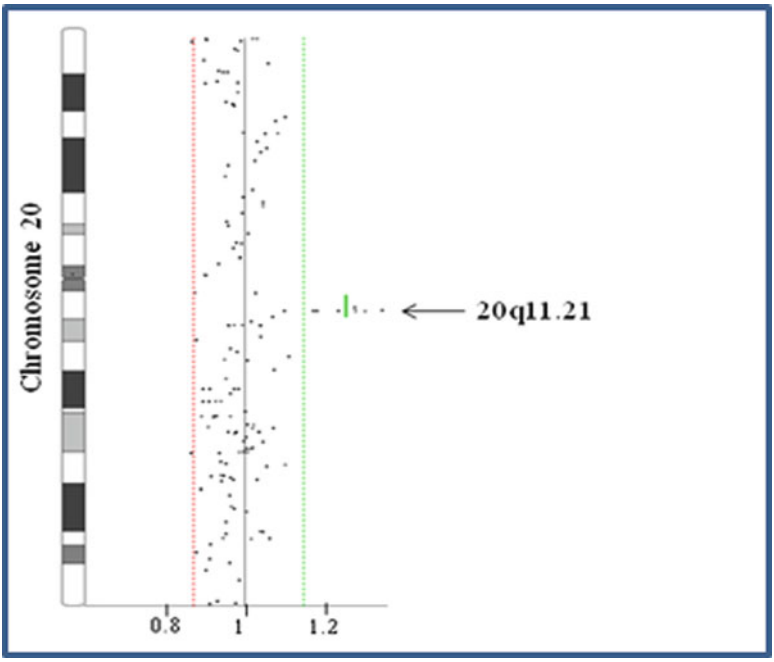

Fig. 9.4 Chromosome 20 analysis by BAC-aCGH method showing amplification at 20q11.21. Each symbol (*solid squares*) corresponds to a BAC; the *dotted lines* indicate the upper and lower thresholds outside which amplification and deletion are detected

9.2.2.3 Next-Generation Sequencing

The next-generation sequencing (NGS) also called massive parallel sequencing (MPS) or high-throughput sequencing (HTS) parallels the sequencing process, producing millions of sequences. Whole-genome sequencing provides a powerful method to identify all genetic changes in cultured cells, including deletions, amplifications, chromosomal translocations, uniparental disomy and single-nucleotide mutations even when a small subpopulation of cells is affected. High-throughput DNA sequencing approaches have allowed further exploration of the genomic integrity of hPSCs. Whole-genome or exome resequencing has provided unprecedented resolution for identifying single base-pair mutation in PSCs (Gore et al. 2011; Ji et al. 2012; Funk et al. 2012; Lafaille et al. 2012; Cheng et al. 2012; Young et al. 2012; Ruiz et al. 2013; Okita et al. 2013). However, most of these studies have focused on somatic mutations occurring in hiPSCs during reprogramming rather than those occurring during subsequent culturing. NGS will surely be a method of choice in the future. However, the cost and the logistics required for the routine use of this technique still prevent this type of application. Another challenge for NGS analysis is to distinguish polymorphisms from true mutational events.

9.2.2.4 Gene Expression-Based Method

A new method for detecting chromosomal aberrations in cultured cells has been proposed based on the principle that regions with biased gene expression are correlated with chromosomal abnormalities (Mayshar et al. 2010; Ben-David et al. 2011). The integrity of stem cells can thus be checked using RNA expression profiles. The meta-analysis of global gene expression of over 400 stem cell lines (NSC, mesenchymal stem cells (MSC), PSC) successfully identified 4 % (MSC) to 9 % (PSC and NSC) of lines with large clusters of genes with significantly higher or lower expression, indicating genomic gains or losses (Mayshar et al. 2010; Ben-David et al. 2011). The main advantages of this method are the possibility to use the same sample for both gene profiling and genomic integrity control and the opportunity to perform retrospective examinations of genetic integrity. However, the sensitivity and the resolution (>10 Mb) are low compared to arrays-based approaches.

9.3 Genomic Alteration in Pluripotent Stem Cells

9.3.1 Origin of Genomic Instability

There are several ways for ESCs and iPSCs to acquire genomic alterations. In human ESCs, genomic abnormalities can have a parental origin (already present in the gametes before the fertilization), or else they can become abnormal during the derivation of the cell line. In iPSCs, genomic variations can be inherited from the parental somatic cells from which the cell lines are originated (already present in a few cells or in the whole population), can result from integration of the reprogramming transgenes or else can arise during the reprogramming process. ESCs and iPSCs also share common sources of genomic instability: the expansion, the freezing and the thawing of the cells.

9.3.2 Chromosomal Defects

The mechanisms involved in maintaining chromosome integrity concern various pathways implicated in the cell division and in the fidelity of DNA replication. Aneuploidies and structural variants originate from distinct mechanisms. Aneuploidies arise when chromosomes fail to segregate properly during cell division. The control of cell division includes mechanisms such as chromosome condensation and segregation, sister chromatid cohesion, kinetochore function, centrosome duplication, telomere and centromere stability and cell cycle progression checkpoint. An alteration in any of these functions is likely to cause defects in mitotic segregation during anaphase.

Since 2004, several studies have reported that chromosomal abnormalities frequently accumulate in prolonged cultures of human ESCs (Draper et al. 2004; Inzunza et al. 2004; Brimble et al. 2004). Gains of chromosome arms or whole chromosomes were the most frequent changes reported mainly due to the karyotyping methods (banding) that were used. Many other studies have since confirmed the propensity of PSCs to acquire chromosomal changes in culture (Mitalipova et al. 2005; Maitra et al. 2005). A few years later, Baker and colleagues listed the abnormal ESC karyotypes observed by different groups (Baker et al. 2007). The most frequent alterations then described were gains of entire chromosomes (12, 17 and X) or gains of chromosome arms (12p, 17q). Aneuploidies of chromosome 12 and 17 are very common in germ cell tumours and embryonal carcinoma (EC) cells and contribute to enhancing the capacity of germ cell tumours, EC cells and ESCs for self-renewal (Andrews et al. 2005). In 2011, a large-scale G-banding study conducted on more than 1,700 PSC samples including 219 iPSC lines (552 samples) and 40 ESC lines (1,163 samples) from 29 laboratories reported 12.5 and 12.9 % abnormal karyotypes, respectively (Taapken et al. 2011). The method used to reprogramme iPSCs (integrating or nonintegrating vector) does not seem to affect the tendency to acquire chromosomal defects (Mayshar et al. 2010; Martins-Taylor et al. 2011; Taapken et al. 2011; Ronen and Benvenisty 2012). These frequencies are quite similar to that observed in Ben-David et al. (2011) (9 % of cell lines carrying large chromosomal defects) but are much lower than those observed in Amps et al. (2011) (34 % for ESCs and 27 % for iPSCs). ESCs and iPSCs share common recurrent chromosomal defects such as duplications of whole chromosomes 8, 12, X or chromosome arm 12p. Trisomy 12 is the most frequent abnormality observed in both cell types (42.6 % of all aberrations detected in ESCs and 31.9 % in iPSCs) (Taapken et al. 2011). Cells carrying a trisomy of chromosome 12 are able to overtake the normal diploid cells in only a few passages (Catalina et al. 2008). It is often suggested that NANOG, located on chromosome 12p, is the master gene which drives the selective advantage of these trisomic cells, but the selective advantage may instead be conferred by the overexpression of a combination of genes (Amps et al. 2011). The high incidence of chromosome 17 duplications widely reported in ESCs is not observed in iPSCs with a few exceptions (Ben-David et al. 2011). This likely reflects the diversity of culture conditions in the PSC laboratories that create different selection and constraint events leading to cell adaptation.

9.3.3 Subchromosomal Defects

Replicative stress and oxidative damage underlie many genomic structural rearrangements (copy number variant (CNV), translocation, inversion) (Arlt et al. 2009). Repeat DNA elements comprise more than 50 % of the human genome. Some of them are mobile within our genome (retrotransposons, transposons) and are also sources of structural variations. Most retrotransposons are inactive and only a few are still active. Among them, active short interspersed elements (SINEs, such

as Alu sequence) and long interspersed elements (LINEs, such as L1 sequences) can lead to a variety of genomic rearrangements (Symer et al. 2002). In humans, L1 retrotransposition occurs in early embryonic development (Kano et al. 2009). Munoz-Lopez and colleagues have demonstrated retrotransposition of transfected L1 in human ESCs (Munoz-Lopez et al. 2012). L1 retrotransposition may occasionally lead to the generation of genomic variability in cultured pluripotent cells by a variety of mechanisms (Wissing et al. 2012).

Thanks to the development of array-based DNA methods, the resolution of genomic integrity analyses has been significantly increased. The first studies reporting subchromosomal regions prone to copy number variations (CNV) have been published in 2008 (Lefort et al. 2008; Spits et al. 2008). These studies identified recurrent amplifications at 20q11.21. Afterwards, high-resolution SNP analysis on 186 PSC samples (130 samples from 86 ESC lines and 56 samples from 37 iPSC lines) reported large regions of CNV in both hESC and iPSC lines randomly distributed along the chromosomes and two regions of recurrent chromosomal instability on chromosome 12p13 (13 % of the ESC lines) and 20q11.21 (10 % and 3 % of the ESC and iPSC lines, respectively) (Laurent et al. 2011). The same study also revealed that during the reprogramming process, deletions are positively selected in iPSC lines and then negatively selected during passaging to the benefit of duplications. At early passages, the mutation load of iPSCs appears to be higher than that observed in ESCs, and during amplification there are no more differences (Hussein et al. 2011). The regions deleted in iPSCs contain genes with tumour-suppressor activity, whereas the duplicated regions contain genes associated with tumorigenicity. More than 90 % of duplications in hPSCs are small size changes undetectable with metaphase-based or gene expression methods (Laurent et al. 2011). The same year another study, published by the International Stem Cell Initiative (ISCI), analysed 125 human ESC lines and 11 iPSC lines from 38 laboratories at early and late passages and determined that more than 20 % of the lines were carrying 20q11.21 amplifications. Currently, the genomic instability of this region has been identified in more than 30 cell lines around the world (Maitra et al. 2005; Wu et al. 2008; Lefort et al. 2008; Spits et al. 2008; Werbowetski-Ogilvie et al. 2009; Elliott et al. 2010; Narva et al. 2010; Laurent et al. 2011; Amps et al. 2011; Martins-Taylor et al. 2011). The size of the duplicated region ranges from 0.5 to 4.6 Mb. The smallest amplified region includes three expressed genes, namely, ID1, BCL2L1 and HM13. Cells carrying 20q11.21 duplication express the archetypal hESC markers but display some features of neoplastic progression, including growth factor independence, increase in the frequency of tumour-initiating cells and aberrant lineage specification (Werbowetski-Ogilvie et al. 2009). In vivo, the 20q11.21 region is amplified in breast carcinomas (Tanner et al. 1996; Guan et al. 1996), lung cancer (Tonon et al. 2005), melanoma (Koynova et al. 2007), hepatocellular carcinoma (Midorikawa et al. 2006), bladder cancer (Hurst et al. 2004) and early-stage cervical cancer (Scotto et al. 2008). The selective advantage conferred to these mutant cells may be driven by a single gene, which could be BCL2L1 (Amps et al. 2011).

To a lesser extent, other subchromosomal regions of genomic instability have been recurrently identified by different groups. In human ESCs, losses of 18q21qter

and gains of 8q24.21 and 1q31.3 regions have been detected (Maitra et al. 2005; Spits et al. 2008; Hovatta et al. 2010; Amps et al. 2011). In iPSCs, gains of 8q24.21 and 1q31.3 regions have also been observed (Elliott et al. 2010; Martins-Taylor et al. 2011).

9.3.4 Point Mutations

Replicative stress, deficient DNA damage response and deficient cell cycle checkpoints lead to the accumulation of genomic changes at the nucleotide level (Hyka-Nouspikel et al. 2012; Hussein et al. 2012). Therefore, point mutations also contribute to this adaptation. Recently, thanks to the development of NGS, whole genomes or exomes of hPSCs have been sequenced (Gore et al. 2011; Ji et al. 2012; Funk et al. 2012; Lafaille et al. 2012; Cheng et al. 2012; Young et al. 2012; Ruiz et al. 2013; Okita et al. 2013). The first exome sequencing of 22 hiPSC lines identified an average of five protein-coding point mutations per line, among which at least half pre-existed in the parental fibroblasts and the remaining occurred during reprogramming (Gore et al. 2011). Another study done on five iPSC lines and their parental cell sources revealed that three quarters of the somatic coding mutations occurred during reprogramming, 7 % during cell expansion and the remaining (less than 20 %) were inherited from the parental cells (Ji et al. 2012). Other authors have suggested that point mutations arose in cultured iPSCs but that most were pre-existing (Cheng et al. 2012; Young et al. 2012). Whole exome sequencing of four iPSC lines and their parental fibroblasts showed more than 99.9 % concordance (Lafaille et al. 2012): at the scale of the whole genome, this represents more than one million discordant nucleotides. Among the exonic variant not common to iPSC lines and their respective parental fibroblasts, some affect cancer-related genes among which are heterozygous variations in the oncogene TP53 (Lafaille et al. 2012).

9.3.5 Mutations in Mitochondrial DNA

The impact of long-term culture on the integrity of mitochondrial DNA (mtDNA) has been poorly explored. Human stem/stromal cells have a relatively stable mitochondrial genome with a mutational load that did not change under hypoxia or after prolonged culture (Oliveira et al. 2012). Opposing observations have been done on human pluripotent stem cells. In 2005, one study screened the entire mtDNA genome of hESC lines using a mitochondrial resequencing oligonucleotide (Maitra et al. 2005) and revealed heteroplasmic mtDNA alterations (a fraction of mtDNA molecules) in two of nine (22 %) hESC lines. Six years later, a study on iPSC lines generated from young healthy donors confirmed, using next-generation sequencing, that hPSCs harboured both homoplasmic (all mtDNA molecules) and heteroplasmic

(Prigione et al. 2011a, b) single nucleotide mtDNA mutations. There are two main sources of genomic alteration of mtDNA in hiPSCs. Mutations may arise during somatic development of human cell sources or from random mutation events upon reprogramming and subsequent culture (Prigione et al. 2011a, b). However, the observed defects were not already present in the somatic cell sources. Using PCR amplification of overlapping fragments and Sanger sequencing, Van Haute et al. (2013) identified a plethora of large mtDNA deletions with an average mutational load of 23 % in 16 hESC lines.

9.4 Genomic Alterations in Differentiated Cells, with Reference to the Central Nervous System

9.4.1 Developing Brain

Mosaic aneuploidy has been reported in the developing human brain. The frequency of aneuploidy in the developing brain tends to approach 30–35 % (Yurov et al. 2007; Devalle et al. 2012). This rate is close to the amount of cells cleared by programmed cell death (PCD) throughout brain development (Yurov et al. 2009). Thanks to PCD, the frequency of aneuploidy in the adult brain is three times less than in the developing brain. Some degree of aneuploidy therefore appears to be natural during neurogenesis. Variation in the neuronal genome may be an important mechanism for neuronal diversity of the normal brain (Yurov et al. 2007). However, aneuploidy is largely correlated with cancer, ageing and other pathologies. A defective clearance of aneuploid cells may contribute to neurological disorders; brains of patients with Alzheimer disease showed three-and fourfold increase of chromosomes 17 and 21 aneuploidies, respectively; brains of patients with ataxia telangiectasia exhibited a twofold increase of random aneuploidy and schizophrenic patients' brains displayed a threefold increase of chromosome 1 aneuploidy (Devalle et al. 2012). Aneuploidy seems, therefore, to be associated with neurological disorders. Beside aneuploidy, another source of brain genomic instability occurs with defects in the nucleotide excision repair (NER) system (Nouspikel 2007, 2008). NER is strongly attenuated in differentiated cells such as neurons. In theory, the attenuation of NER should not have consequences in post-mitotic cells. Nevertheless, neurons may inappropriately re-enter the cell cycle, and NER attenuation may contribute to genomic instability leading to neuronal loss in various neurological diseases (Yang et al. 2001).

9.4.2 Adult Neural Stem Cells

Chromosomal changes have been reported in cultured mouse fetal-derived NSCs (Diaferia et al. 2011). Trisomy of chromosome 1 was one of the most common

abnormalities observed. Few studies have focused on this question in the humans. By karyotyping 21 human NSC lines derived from different fetal sources, Sareen et al. discovered that 24 % of them displayed trisomy 7 after 7–15 weeks in culture (Sareen et al. 2009). A trisomy of chromosome 19 was also observed in 5 % of the cultured NSC lines following 15–25 weeks. In one NSC line, these two chromosomal defects were present simultaneously. In a large-scale a posteriori gene expression data sets study, Ben-David et al. analysed 58 NSC samples from diverse sources and reported a frequency of 9 % aberrations (Ben-David et al. 2011). They observed a trisomy of chromosome 10 and aneuploidies of chromosome 18. They also confirmed the trisomies of chromosomes 7 and 19 previously described in the original study (Sareen et al. 2009).

9.4.3 PSC-Derived Neural Progenitors

In contrast to pluripotent stem cells, there has been little unravelling of chromosomal abnormalities in their neural derivatives, and this despite the fact that neuronal genomic abnormalities are observed in the developing brain. Two studies reported a stable karyotype in neural derivatives of pluripotent stem cells that were extensively propagated (Koch et al. 2009; Nemati et al. 2011). However, these studies relied on G-banding karyotyping, whereas some—though not all—of the abnormalities may require more detailed techniques to be revealed. At odds with these observations, nonetheless, were the results of three reports that identified an unstable karyotype in neural derivatives of pluripotent stem cells (Ben-David et al. 2011; Varela et al. 2012; Corrales et al. 2012). An a posteriori analysis of hESC-derived NSC samples from several independent laboratories using the gene expression-based method identified aberrations typical of both NSCs (trisomy 19) and PSCs (trisomy 20) (Ben-David et al. 2011). However, the typical PSC aberrations probably arose prior to the differentiation of the hESC. Corrales at al. also described hPSC-derived neuroprogenitors carrying CNVs. Some of these CNVs were inherited from the parental human PSCs whereas the others were detected only in NPCs. However, considering the low sensitivity of aCGH, CNVs already present below aCGH sensitivity may have been selected from the PSC source (e.g. 20q11.21) during or after the neural induction. Finally, in these two studies it is difficult to distinguish anomalies that emerged during neural induction or during the amplification of NSCs from those already present in the PSCs. Moreover, the authors did not assess the in vivo potential of the abnormal cells in xenotransplant models. In contrast, in a third study (Varela et al. 2012), a systematic analysis of genomic integrity of NSCs over time identified a recurrent chromosomal abnormality that affected neural derivatives of hESCs over 20 passages, and was never found at earlier passages. This chromosomal defect, namely, the duplication of the long arm of chromosome 1 (1q) accompanied by a translocation of this supernumerary arm to another chromosome (random), has already been described in haematological malignancies under the name “jumping translocation” and sometimes in solid tumours (breast cancer,

hepatocellular carcinoma, retinoblastoma, paediatric brain tumours) (Fournier et al. 2010; Faria et al. 2010). Cell behaviour of around half of the mutant NSC lines was heavily biased towards self-renewal, and then 1q duplication led to the alteration of the neuronal differentiation potential in vitro. In one NSC line, the in vivo potential has been assessed and abnormal cells were unable to form tumours in rats (Varela et al. 2012). Nevertheless, tests for tumorigenicity in large or small animals (rodents) are meaningful only if positive because many human tumours will not produce tumours when injected into rodents. Thus, the duplication of the 1q arm provides a massive advantage, resulting in the selection of abnormal cells both in cancer and in cultured cells. In the absence of 1q duplication, other chromosomal changes such as polyploidy were observed after long-term culture (Varela et al. 2012).

9.5 Challenges Created by Genome Instability for Cell Therapy

9.5.1 Tumorigenicity of PSC-Based Grafted Cells

The use of PSC derivatives raises safety concerns. Human PSCs offer a limitless cell source for regenerative medicine, but their unlimited self-renewal property bears the risk of tumour formation after engraftment. This concern led the FDA to place a clinical hold on Geron's clinical trial (the hESC spinal cord injury therapy) after half of the animals treated in preliminary studies developed cysts. Due to this adverse effect, the first clinical trial was postponed nearly a year. Uncontrolled proliferation may be due to the persistence of undifferentiated PSC in the graft-giving rise to teratomas or teratoma-like masses. The over-proliferation of non-pluripotent cells is also a concern in cell therapy. Xenograft experiments of human neural progenitors into the rat brain showed signs of overgrowth, suggesting that the path to use in humans requires a way to switch them off after grafting (Aubry et al. 2008). Curative measures may be taken such as the use of antimitotic drugs treatment or suicide gene safety systems (Cohen et al. 2001). The risk of uncontrolled proliferation may potentially be exacerbated by the presence of genomic abnormalities.

9.5.2 Effect of In Vitro Environmental Conditions

9.5.2.1 Cell Culture Time

PSCs are not physiological cells. iPSCs have been induced to pluripotency, and ESCs are maintained indefinitely in a state that is lost physiologically after the blastocyst stage (5.5–7.5 days) in vivo. Compared to any other cell types grown in vitro (mesenchymal stem cells, haematopoietic stem cell, fetal neural cells, etc.) or any

other cells in the body, PSCs accomplish a huge number of cell divisions. In vivo, the rate of spontaneous mutations in normal human cells is about 10^{-7} to 10^{-8} per nucleotide per cell division (Nachman and Crowell 2000; Kondrashov 2003). In vitro, the rate of spontaneous mutation is about 10^{-6} to 10^{-8} per nucleotide per generation in human cell lines (Kuick et al. 1992). In humans, there are approximately 3×10^9 nucleotides per haploid genome, which means between 30 and 3,000 mutations per cell at each cell cycle. Chromosomal defects are observed even in low passage cultures, but this rate increases with passages. Therefore, minimizing cell culture duration is crucial.

Guidelines and standards providing recommendations are lacking (Bai et al. 2013). In 2006, the European Medicines Agency (EMEA) established guidelines on human cell-based medicinal products. The guideline EMEA/CHMP/410869/2006 determined that "the duration of cell culture and maximum number of cell passages should be clearly specified and validated". With adult stem cells this maximum number can be established based of the knowledge of Hayflick limit (Hayflick 1965). With PSCs, it is quite different since these cells do not obey the Hayflick limit.

9.5.2.2 Culture Conditions

Aneuploidies of chromosomes 12 and 17 are observed in both PSCs and EC cells, while amplification of chromosome 20q is detected in either PSCs or in non-seminomatous germ cell tumours that contain EC cells. This may indicate that the selective advantage is related to the pluripotency itself and is not dependent on culture conditions (Amps et al. 2011). However, numerous culture parameters may predispose PSCs to chromosome instability, namely, (1) the cell population size, (2) the culture medium, (3) the method used to passage PSC colonies, (4) the presence versus absence of feeders, (5) the oxygen tension and (6) freezing/thawing.

1. Genetic changes may modify gene expression profiles of the mutated cells and provide them with a selective advantage for self-renewal. This selective advantage can be an increased survival or an increased rate of proliferation. A Monte Carlo simulation model predicted that maintenance of cells in small populations reduces the likelihood that abnormal cultures will develop (Olariu et al. 2010).
2. There may be essential components present in fetal calf serum (FCS) that are lacking in the widely used knockout serum replacement (KSR), making the cells more vulnerable to a wide range of factors. Conversely, KSR contains ascorbate which triggers CD30 expression through an epigenetic mechanism and provides transcriptome changes and a survival advantage that may help hESCs to adapt to in vitro culture conditions (Chung et al. 2010). Moreover, addition of high levels of antioxidants to the medium unexpectedly increases aneuploidy in stem cell cultures (Li and Marban 2010).
3. Passaging of cell lines with enzymatic treatment (collagenase, dispase, trypsin) was believed to generate more abnormalities than mechanical cutting (Buzzard

et al. 2004; Mitalipova et al. 2005). Nevertheless, a large-scale study on more than 180 PSC samples revealed no correlation between passage method (enzymatic vs. mechanical cutting) and the number of CNVs (Laurent et al. 2011).
4. The frequency and the types of karyotypic abnormalities are not affected by the substrates on which PSCs are cultured (Taapken et al. 2011).
5. Hypoxia has been shown to reduce genomic instability and enhance human ESC clonal recovery (Forsyth et al. 2006).
6. Cryoprotectants such as dimethylsulphoxide affect the epigenetic profile of the cells as opposed to trehalose, which preserves cellular integrity (Eroglu et al. 2000; Guo et al. 2000).

9.6 Conclusions

Standards or guidelines to qualify a PSC line as acceptable or not for cell therapy are definitely needed. Genomic instability is a hallmark of cancer. Prior to any cell therapy, it is therefore crucial to check genomic integrity of the cells intended to be grafted (Hanahan and Weinberg 2011; Goldring et al. 2011). The guideline EMEA/CHMP/410869/2006 determined that it is required to test chromosomal integrity and tumorigenicity of cells derived from a cell culture. However, there is no consensus on the method(s) that should be used to qualify PSC lines. Genomic integrity quality control of the clinical RPE lot (ACT trial) has been evaluated by karyotyping 20G-banded metaphases and 200 interphase nuclei assayed by FISH for chromosomes 12 and 17 (Schwartz et al. 2012). Since some chromosomal defects are not detectable with G-banding, detailed genetic profiling should be undertaken before engraftment into the patient (Stephenson et al. 2010; Goldring et al. 2011). A solution would be to associate at least two methods that combine resolution and sensitivity (G-banding and DNA-based technologies such as SNP arrays or NGS). The interpretation of DNA-based analyses is complex because there is no means to assess the impact of genomic variation on the functionality or on the tumorigenicity of cultured cells. However, aneuploid cultures or cell lines carrying recurrent chromosomal defects as well as mutations already reported in cancers should be excluded (Bai et al. 2013) to optimize the safety of the therapy.

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Chapter 10
Links Between Injury-Induced Brain Remodeling and Oncogenesis

Elias A. El-Habr and Marie-Pierre Junier

Abbreviations

8-OxoG	8-Oxoguanine
Ascl1	Achaete–scute complex-like 1
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
bFGF	Fibroblast growth factor 2 (basic)
BRAC1	Breast cancer 1
BRCA2	Breast cancer 2
Brn2a	Brain-2
CDK	Cyclin-dependent kinase
chk1	Checkpoint kinase 1
chk2	Checkpoint kinase 2
CNS	Central nervous system
CNV	Copy number variations
CSC	Cancer stem cells
DSBs	DNA double-strand breaks
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ESCs	Embryonic stem cells
GFAP	Glial fibrillary acidic protein

E.A. El-Habr • M.-P. Junier (✉)
French Institute of Health and Medical Research U1130, French National Center for Scientific Research, Pierre-and-Marie-Curie University, Paris, France
e-mail: marie-pierre.junier@inserm.fr

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3_10,

GSC	Glioma stem cells
H3K27me	Histone H3 lysine 27 methylation
H3K4me	Histone H3 lysine 4 methylation
H3K9me	Histone H3 lysine 9 methylation
HB-EGF	Heparin-binding EGF-like growth factor
HR	Homologous recombination
iPSCs	Induced pluripotent cells
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
mTOR	Mammalian target of rapamycin
Myt1l	Myelin transcription factor-like 1
NEIL1	Endonuclease VIII-like 1
NEIL3	Endonuclease VIII-like 3
NF1	Neurofibromin 1
NHEJ	Nonhomologous end-joining
NPC	Neural progenitor cells
NSC	Neural stem cells
OGG1	8-Oxoguanine DNA glycosylase
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma
RCAS-TVA	Replication-competent avian sarcoma-leukosis virus splice acceptor-avian receptor tv-a
RTK	Tyrosine kinase receptors
SSA	Single-strand annealing
Shh	Sonic hedgehog
TGFα	Transforming growth factor alpha

10.1 Introduction

Proper functioning of the CNS is characterized by its ability to adapt to a wide array of rapidly varying situations. This adaptation extends to pathology. Remodeling of spared neural circuits has been repetitively observed to partly mitigate the deleterious consequences of various brain injuries, especially in children. Research over the past 10 years has shed new light on the cell types and the molecular circuits that could participate in the endogenous regenerative properties of neural tissues. The explosion of studies on postnatal and adult neurogenesis has unraveled an unsuspected variety of neural stem cells (NSC) and neural progenitors (NPC) in neurogenic and possibly in non-neurogenic areas of the CNS. The molecular mechanisms underpinning the maintenance of their properties and governing their differentiation along the different neural lineages have been extensively explored. In parallel, the discovery in 1999 of NSC with some characteristics of astrocytes (Doetsch et al. 1999) has led to the reexamination of the plastic capabilities of these glial cells,

resulting in the finding that reactive astrocytes that develop following brain injury can be reoriented towards a neurogenic state.

The identification of potential cellular and molecular players in neural tissue repair has renewed the hope of achieving new strategies aimed at harnessing in a targeted manner the endogenous repair potential of the CNS following invasive traumatic injuries or neurodegeneration of genetic origin. These therapeutic strategies range from expanding the NSC/NPC pools, manipulating their differentiation fate, to redirecting glial cells towards a multipotent state.

These hopes are, however, counterbalanced by results derived from studies on the development of brain glial neoplasms, which have pinpointed several common features between brain cancer cells, developing neural cells of the immature and mature brain and/or injured nervous tissues.

Profound tissue rearrangements due to gliosis and neo-angiogenesis take place following brain injury. They are characterized by the reacquisition of immature features such as proliferation, upregulation of signaling pathways normally active in embryonic tissues, and recruitment of dormant NSC/NPC. Similarly, development of brain neoplasms is accompanied with the setting up of a novel tissue per se, composed of diverse cancerous and normal cell types that interact with each other. Several studies have pointed to the essential role sustained by these interactions for the maintenance and growth of the tumors, most of the underlying mechanisms being also mobilized upon brain injury (Christie and Turnley 2012; Clark et al. 2007; Sofroniew and Vinters 2010). The process of glioma development, like the process of remodeling injured nervous tissue, is thus accompanied with not only aberrant cell proliferation and survival but also changes in cell differentiation, as seen for other cancers (Harris 2004).

In addition, the parallels drawn by several investigators between neurogenesis and neurooncogenesis have led to the identification within some brain neoplasms of cancer cells endowed with functional and molecular properties similar to those of NSC or NPC, the so-called cancer stem cells (CSC). These cells divert molecular circuits normally used by NSC, NPC, and even reactive astrocytes. Intense debate is still ongoing to determine whether these cells are at the origin of the tumor and/or are the result of its evolution. The cell of origin of brain neoplasms remains a matter of speculation because of the complexity of the tumor tissue and the current impossibility to identify a preneoplastic state in the brain (Collins 2004). Nevertheless, distinct data sets indicate that brain neoplasms are likely to be derived from neural cells endowed with immature features either innate or acquired. They thus show that some of the cells that represent the most likely targets for strategies aimed at boosting the endogenous repair abilities of the brain are also the most prone to cancerous transformation.

Although brain neoplasms of traumatic origin are difficult to identify, several reports have documented such occurrences following traumatic injury (Moorthy and Rajshekhar 2004) or in association with pharmacoresistant focal epilepsies (Becker et al. 2006). These findings further reinforce the need to cross-examine the data derived from the studies of neurogenesis and oncogenesis in order to identify and circumscribe the possible risks of regenerative medicine anchored on harnessing the repair potential of the brain. In the following paragraphs, we have attempted

to highlight the behaviors of neural cells in the context of injuries that could be the source of a cancerous transformation.

Cancerous transformation is classically viewed as a disarray of inhibition of cell cycle and apoptotic signals, as well as problems in cell differentiation, that result in enhanced genomic instability, a key characteristic of cancer cells.

Injury to nervous tissues initiates a complex array of phenomena into which glial cells play a central role. This gliosis recruits cells of the hematopoietic lineage (microglia, lymphocytes, and macrophages) and cells of the neural lineages (NG2 cells, astrocytes, neural stem cells, and neural precursor cells). The extent as well as the transient or permanent character of this reaction depends on the severity of the initial insults, the most severe of which (traumatic brain injury, stroke, and other injuries to the blood–brain barrier) results in the formation of a glial scar restoring the isolation of the CNS (Ridet et al. 1997). This process is underpinned by a large array of changes particularly in astrocyte, progenitor, and stem cell behaviors. These changes are characterized by the transient reacquisition of features usually associated with cancer cells, i.e., signs of genomic perturbations, reentrance into the cell cycle, and troubles in cell differentiation usually described in this setting by the term "plasticity." All these changes can be viewed as intrinsic neoplastic features of neural cells reacting to nervous tissue injuries.

10.2 Genomic Instability

Cancer cells are first characterized by their genomic instability, which allows them to acquire specific properties such as escape from the inhibitory control of cell cycle and enhanced resistance to death-inducing signals (Hanahan and Weinberg 2011). This genomic instability can be prompted by different genomic alterations ranging from anomalies in chromosome numbers (aneuploidy), duplication or deletion of DNA fragments (copy number variations, CNVs), to single nucleotide alterations linked to errors in DNA repair.

10.2.1 Molecular Basis of Genomic Variation

The molecular bases of genomic variation are multiple (see for review Holland and Cleveland 2012; Sulli et al. 2012). First explored in cycling cells, they notably involve molecules required for cell cycle check points (ATM, ATR, chk1, chk2) (Cimprich and Cortez 2008; Reinhardt and Yaffe 2009; Taylor and Byrd 2005) and the tumor suppressor genes p53 and Rb, the inactivation of which induces aneuploidy (Hernando et al. 2004; Holland and Cleveland 2009; Talos and Moll 2010). Structural modifications of DNA are not restricted to cells undergoing DNA replication. They extend to quiescent/postmitotic cells, which are exposed to a broad range of genotoxic stresses even in physiological conditions. Repair of DNA mobilizes different DNA damage response pathways according to the type of DNA lesion. Nucleotide lesions caused by oxidation are removed by the base-excision pathway,

which is initiated by DNA glycosylases recognizing the modified base (Robertson et al. 2009). Repair of DNA double-strand breaks (DSBs) can be achieved through at least 3 pathways. Nonhomologous end-joining (NHEJ) acts in the absence of sequence homology and involves the joining of nonhomologous DNA ends, whereas single-strand annealing (SSA) occurs at sequence repeats. Of note, repair errors are more frequent following NHEJ and SSA than homologous recombination (HR), which is mobilized when an intact DNA strand may serve as a repair template and is only active in the S and G2 phases of the cell cycle (reviewed in Moynahan and Jasin 2010).

10.2.2 Genomic Variation in Normal Neural Tissues

Although the study of genomic variation in the normal brain is very recent, it has been documented to be not only an integral part of the normal developmental processes but also of the normal functioning of the adult CNS. Abnormal numbers of aneuploid cells have in addition been associated with neurological disorders as discussed by N. Lefort and M. Peschanski in Chap. 9 of this book. Variations in chromosome number (aneuploidy) have been documented in cerebral cortex neurons of wild-type mice (Rehen et al. 2001). These somatic changes in chromosome numbers, where hypoploidy predominates, confer to the brain a mosaic aspect where normal euploid cells coexist with aneuploid cells. This observation was extended to both neuronal and glial cells of the normal human brain (Kingsbury et al. 2005; Pack et al. 2005; Rehen et al. 2001, 2005; Yang et al. 2003; Yurov et al. 2005, 2007). Probing chromosome 21, Rehen and colleagues reported that chromosome 21 aneuploid cells represent around 4 % of the estimated total cell numbers of the human brain, whereas the rate of chromosome 21 aneuploidy in interphase lymphocytes was estimated at 0.6 % (Rehen et al. 2005). Indirect evidences have been provided for a functional integration of aneuploid cells in adult neuronal networks (Kingsbury et al. 2005), although aneuploidy is not without consequences on the functioning of the cell. Gene expression is altered by chromosome loss in mouse neural cells (Yang et al. 2003), and proliferation and metabolism are affected in fibroblasts derived from mice carrying an extra copy of a chromosome (Williams et al. 2008). Consistent with these observations, a recent study indicates that only moderate levels of aneuploidy (less than five gain or loss of chromosomes) are tolerated by neural cells and contribute to adult brain patterning (Peterson et al. 2012). In the mouse embryonic and postnatal germinal zones of the forebrain, 15–33 % of the proliferating cells of the brain are aneuploid (Rehen et al. 2001, 2005; Westra et al. 2008). Most aneuploid neural cells are eliminated through apoptosis. Pharmacological blockade or genetic deletion of the apoptosis effectors caspase-3 or caspase-9 increases neural cell numbers with severe aneuploidy (Peterson et al. 2012). A recent study revealed neuronal activity as an additional—and unexpected—potential source of genomic variations in postmitotic cells of the normal brain. Transient DNA DSBs, repaired within a day, were observed in neural networks of mice responding to exploration

of novel environments and to optogenetic stimulations (Suberbielle et al. 2013). Altogether, these studies coupled with the demonstration by F. Gage's laboratory of somatic insertions of L1 retrotransposons in the mouse and human brains (Muotri et al. 2005; Muotri and Gage 2006) suggest that genetic mosaics of various origins contribute to shape the brain of each individual in a unique manner. Whether or not this genomic variability translates into functional benefits is an open question.

10.2.3 Genomic Variation and Instability in Injured Neural Tissues

Genomic modifications can result in genomic instability and ultimately in pathological genomic alterations. The deleterious effects of genomic instability are well recognized. Genomic alterations are a key feature of cancer cells and of several neurological diseases (Biesecker and Spinner 2013; Bras et al. 2012; McKinnon 2009). Several studies favor the possibility that genomic instability can be promoted in the injured brain. Oxidative stress is a prime event in several neurodegenerative diseases as well as in traumatic brain injury and in hypoxia-ischemic lesions and has been repetitively associated with increased levels of DNA damage in these pathological settings (Smith et al. 2013). Mobilization of DNA repair pathways is an intrinsic component of neural brain tissue response to injury. 8-Oxoguanine DNA glycosylase (OGG1), responsible for the excision of 8-OxoG, is upregulated following MCAO, and OGG1-deficient mice have increased infarct volume after cerebral ischemia (Liu et al. 2011). Likewise, lack of the DNA glycosylase NEIL1 (endonuclease VIII-like 1), which also excises oxidatively modified nucleotide bases, aggravates brain lesions induced by ischemic stroke (Canugovi et al. 2012). Another DNA glycosylase, NEIL3 (endonuclease VIII-like 3), has been shown to be essential for the promotion of neurogenesis that follows ischemic insults in mice (Sejersted et al. 2011). Most interestingly, NSCs derived from OGG1 −/− mice exhibit increased mitochondrial DNA damages during their differentiation and are biased towards adoption of an astrocyte phenotype (Wang et al. 2011). Appropriate DNA repair thus appears essential for proper NSC/NPC behavior not only during development but also during the adult stage. It might also prove to be an essential component of the response of postmitotic cells to injury. Repair of DNA DSBs occurring in response to normal brain behavior has been shown to be seriously impaired in mice expressing the human amyloid precursor protein (Suberbielle et al. 2013), a finding consistent with previous observations of increased levels of aneuploid cells in Alzheimer diseased brain (Arendt 2012; Iourov et al. 2009). Evidences for induced DSBs in neurons in response to sublethal activation of ionotropic glutamate receptors have been observed in neuron cultures (Crowe et al. 2006). The existence of genomic variability in the human brain and hence of potential genomic instability is thus likely to be an issue to address in the context of regenerative repair. Forced exit of NSC from quiescence may in particular sensitize

the cells to cancerous transformation. Lengthening of the cell cycle has been suggested as one way for cancer cells to allow increased time for DNA lesion removal (Frosina 2010). On the other hand, self-renewing cells such as ESCs have been shown to progress from G1 to S phase of the cell cycle despite DNA lesions (Aladjem et al. 1998; Hong and Stambrook 2004), a phenomenon that could be linked in part to defective nuclear translocation of p53 (Aladjem et al. 1998). Moreover, a recent study questioning the origin of genetic anomalies in induced pluripotent cells (iPSCs) showed that reprogramming performed with ectopic factors and not with viruses induces DNA DSBs, regardless of the method used for reprogramming. Intriguingly, the same authors discovered that efficient reprogramming depends on members of the homologous recombination pathway such as BRCA1, BRCA2, and RAD51 (Gonzalez et al. 2013). Altogether these different studies indicate that the DNA repair pathways could be intrinsically intermingled with cell fate determination. Manipulation of the endogenous repair potential of neural tissues, especially using strategies targeting NSC/NPC or aiming at boosting the reprogramming potential of reactive glial cells (see Sect. 10.4) is thus likely to face unexpected challenges, further complicated by the discovery that the brain is not comprised of genetically identical cells. This underlies the need for extending research aimed at identifying in a precise manner the level of genomic instability triggered by different brain injuries, especially in cycling cells, the molecular pathways that underpin these variations, and at determining the eventual beneficial or detrimental roles they sustain in the final outcome of the neural tissues to injury.

10.3 Proliferation

Although tumors are no longer viewed as simple bulks of cancer cells proliferating in an anarchic manner, alleviations of the inhibitory control on cell cycle entrance remains an essential feature of cancer cells.

10.3.1 Proliferation in the Normal and Injured CNS

The CNS contains only a few mitotically active neural cells outside areas of neurogenesis, and the molecular basis of this restriction of the cell cycle is poorly defined. In physiological conditions, most cycling cells correspond to NG2 cells. In the adult rodent cortex and spinal cord, NG2 cells represent 50–75 % of all bromodeoxyuridine-incorporating cells (Dawson et al. 2003; Horner et al. 2000) and exhibit a slow turnover rate (Dawson et al. 2003; Levine et al. 1993). In the adult human cortex and white matter, they represent the largest pool of cycling cells (Fig. 10.1), most of them being in the early G1 phase of the cell cycle (Geha et al. 2010). Contrary to NG2 cells, astrocytes are not mitotically active in the normal brain. Injury alleviates

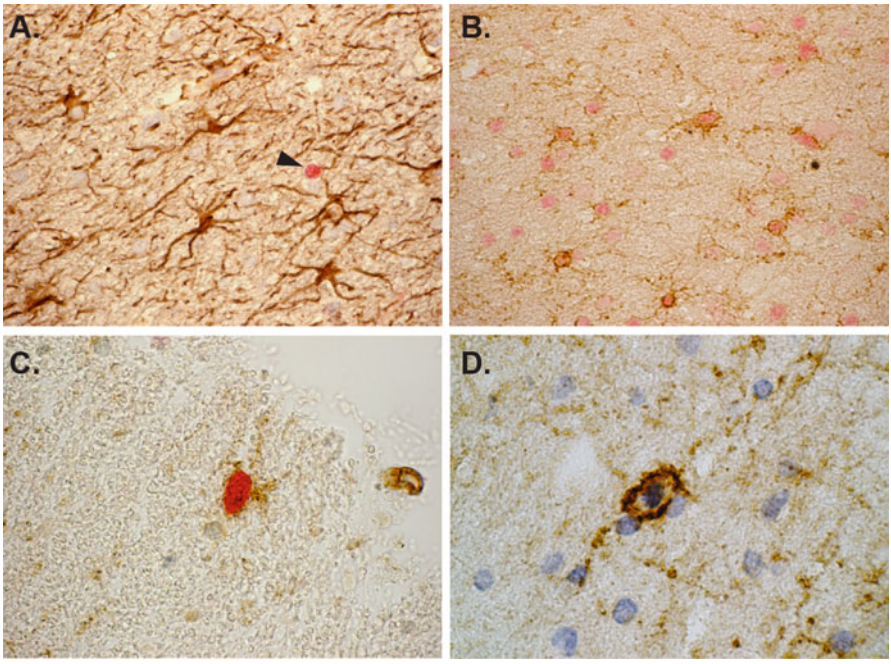

Fig. 10.1 Cycling cells in the adult human cortex correspond to NG2-expressing cells. Cycling cells were identified with Ki-67/Mib-1 expression. (**a**) No Mib-1-positive cells (*red, arrow*) co-express GFAP (*brown*). (**b**) Double immunostainings showing co-expression of Olig2 (*red*) and NG2 (*brown*) (**c**). All Mib-1-positive cells (*red*) co-localized with non-endothelial NG2-positive cells (*brown*). (**d**) Illustration of an NG2-positive cell (*brown*) undergoing mitosis. Magnification: in **a** and **b**, ×200; in **c** and **d**, ×400. In **a**, **c**, **d**: Hemalun counterstaining. Microphotographs kindly provided by Dr. Pascale Varlet. For further information, see Geha et al. 2010

the usual restraint of cell cycle observed in the CNS, NG2 cells, and astrocytes reentering the cell cycle, whereas NPC/NSC exhibit enhanced proliferation.

Brain injuries ranging from open wounds to inflammation have been shown to trigger NG2 cell proliferation in rodents at the injured site (Levine 1994; Nishiyama et al. 1997, reviewed in Dawson et al. 2000). Likewise, enhanced numbers of NG2 cycling cells have been described in human patients suffering from non-lesional pharmacoresistant mesial temporal lobe epilepsy (Geha et al. 2010). The fate of NG2 cells following brain injury is discussed by J.M. Mangin in Chap. 7 of this book. Astrocytes also reenter the cell cycle following brain injuries, albeit the proportion of reactive astrocyte resuming proliferation can vary from 1 to 50 %, according to the type of injury inflicted, and to their distance from the initial site of damage (Bardehle et al. 2013; Buffo et al. 2008; Sirko et al. 2013; Sofroniew 2009). The origin of these dividing reactive astrocyte—either mature astrocytes reentering the cell cycle or astrocytes derived from glial progenitor—has long been debated (reviewed in Sofroniew and Vinters 2010). Recent work, funded on genetic

cell fate mapping in vivo has provided convincing evidence that proliferating reactive astrocytes developing upon cortical stab wound injuries originate from quiescent astrocytes of the parenchyma (Buffo et al. 2008). The same team, using 2-photon imaging in live mice, has further identified a preferential localization of these proliferating astrocytes next to vascular elements (Bardehle et al. 2013). NSC/NPC constitute additional neural cells that may respond by enhanced mitosis to injury (Aguirre et al. 2010; De Filippis and Delia 2011; Gao and Chen 2013; Itoh et al. 2011, 2013; Rasmussen et al. 2011; Sanin et al. 2013). The response of NSC/NPC to traumatic brain injury is reviewed by Hong and colleagues in Chap. 4 of this book.

Multiple signaling pathways have been shown to participate in the initiation and/or development of gliosis, according to the type and localization of the injury. The origin of these varied molecular signals can range from distress signals emitted by injured and dying neurons to plasma elements reaching the neural parenchyma upon blood–brain barrier rupture. This diversity in cell sources is matched by the wide spectrum of molecules that can affect glial reactivity.

10.3.2 Common Signalling Pathways Controlling Gliosis and Glioma Development

The pathways most repetitively found to be upregulated following all types of injuries are also the ones known to play essential roles in the development of glial tumors (Fig. 10.2). The EGFR signaling pathway provides one of the most thoroughly studied examples of the growth factor pathways involved in both the control of gliosis and of glioma development, primarily through its mitotic effects.

The tyrosine kinase receptor EGFR and its ligands (EGF, TGFα, HB-EGF, amphiregulin) are found at low levels in populations of astrocytes, neurons, and NSC/NPC in the embryonic and adult CNS. EGFR activation exerts mitogenic or differentiation actions at different steps of the pathway that leads from an NSC to a mature astrocyte. During the late stages of embryonic development, it favors the preferential differentiation of progenitors into astrocytes over neurons, an effect mediated by enhanced EGFR expression, and segregation into the daughter cell that will acquire an astrocyte identity (Sun et al. 2005). Differentiated astrocytes remain sensitive to EGFR activation. TGFα acts as a gliatrophin for rodent and human astrocytes, as well as for drosophila glia cells (Beck and Fainzilber 2002) promoting their growth as well as their survival (Sharif et al. 2006). Accordingly, mutant mice defective in TGFα or EGFR expression present reduced numbers of astrocytes at the adult age (Kornblum et al. 1998; Sibilia et al. 1998; Weickert and Blum 1995) and enhanced astrocyte apoptosis (Wagner et al. 2006). Like astrocytes, NSC and NPC (including the NG2 progenitor population) remain responsive to EGFR signaling from development to adult ages, responding notably to EGFR activation by enhanced proliferation (Aguirre and Gallo 2007; Craig et al. 1996; Doetsch et al. 2002;

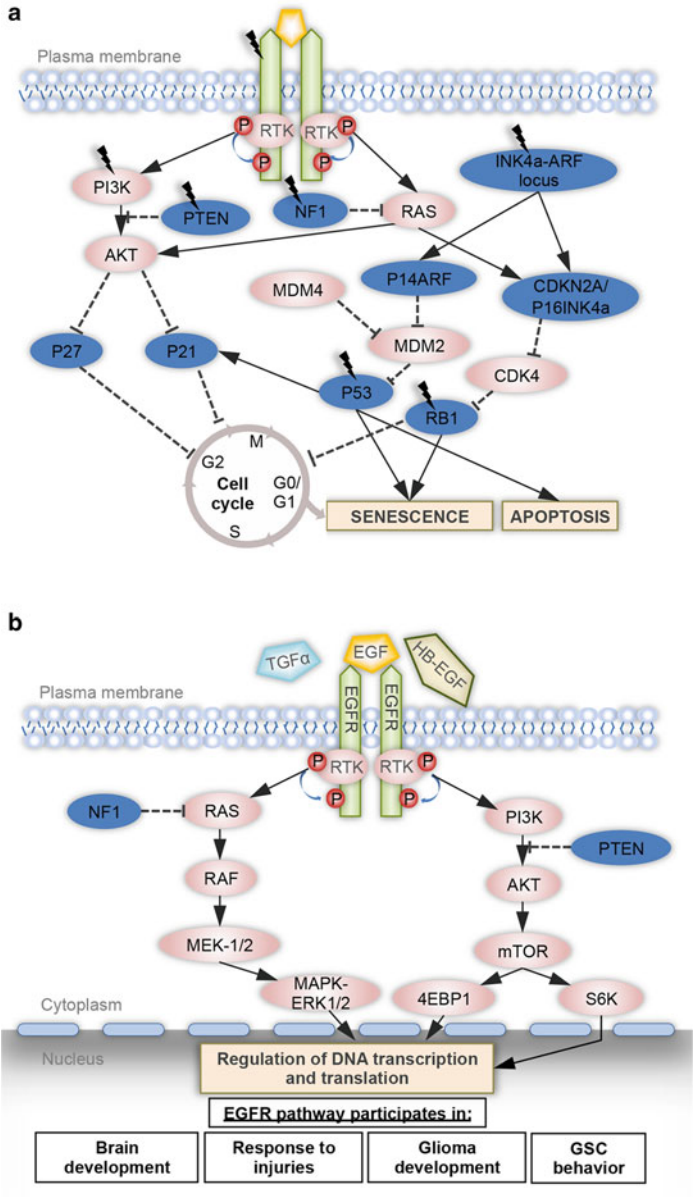

Fig. 10.2 Examples of common signaling pathways controlling gliosis and glioma development. (**a**) Schematic representation of the signaling pathways most frequently deregulated in human glioma. Genes whose activation or inhibition is associated with glioma are depicted in rose and blue, respectively. Genes found to be mutated in human glioblastoma are highlighted with a *thunderbolt symbol*. (**b**) Schematic representation of the EGFR signaling pathway. See text for further details. CDK, cyclin-dependent kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HB-EGF, heparin-binding EGF-like growth factor; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; mTOR, mammalian target of rapamycin; NF1, neurofibromin 1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PTEN, phosphatase and tensin homolog; Rb, retinoblastoma; RTK, tyrosine kinase receptors; TGFα transforming growth factor alpha

Enwere et al. 2004; Ivkovic et al. 2008; Kuhn et al. 1997; Reynolds and Weiss 1992; Tropepe et al. 1999; Weickert and Blum 1995). Upregulation of EGFR ligands (notably TGFα) in astrocytes or neurons accompanied with upregulated expression of EGFR in astrocytes is a common response of the CNS to injury (Birecree et al. 1988; Ferrer et al. 1996; Jin et al. 2002; Junier et al. 1993; Lisovoski et al. 1997; Liu and Neufeld 2004; Nieto-Sampedro et al. 1988; Planas et al. 1998) and is sufficient to trigger astrogliosis throughout the neural axis (Isono et al. 2003; Rabchevsky et al. 1998).

Large-scale genomic characterization of glioblastoma, the most malignant form of glioma, has disclosed mutation/amplification in EGFR and the cognate genes of its signaling pathway (i.e., NF1, RAS, PTEN, AKT, PI3K in over 80 % of the tumors analyzed, Cancer Genome Atlas Research 2008), 45 % of them exhibiting activating mutations in and/or amplification of the EGFR gene. Deregulation of EGFR signaling may thus be reduced to an overexpression of EGFR and/or its ligands with no gene amplification or mutation (Bredel et al. 1999; Ekstrand et al. 1991; Maxwell et al. 1991; Saxena and Ali 1992), TGFα overexpression being, for example, found in about 80 % of all grades of gliomas (Junier 2000). The functional consequences of the autocrine/paracrine loop formed by EGFR and its ligands in glioma cells appear similar to those found for EGFR activation on astrocytes, directly affecting the innate properties of transformed cells, proliferation, and migration/invasion (Engebraaten et al. 1993; Kurimoto et al. 1994; O'Rourke et al. 1997; Pedersen et al. 1994; Tang et al. 1997). Taken together, these results show that dysregulated EGFR signaling is a very frequent event in gliomas and contributes to tumor development. In addition, isolation of cells with stemlike properties from gliomas relies on the availability of EGFR ligands, EGFR activation being an essential regulator not only of GSC proliferation but also of the maintenance of their stemlike and tumorigenic properties (Galli et al. 2004; Mazzoleni et al. 2010).

In this context, the mobilization of the EGFR pathway may constitute one of the molecular mechanisms underlying the acquisition of preneoplastic features observed in neural cells following brain injuries. Numerous efforts have been devoted over the past 10 years to the modeling of gliomas in vivo. Genetic manipulations that allow for the mimicking of the changes in oncogenes and/or in tumor suppressing genes identified in human gliomas have shown that dysregulated expression of EGFR or cognate genes of its signaling pathway at least in immature mice may participate in the cancerous transformation of NSCs or NPCs, including NG2 progenitors (for a complete review of mouse models of gliomas, see Chen et al. 2012).

10.4 Phenotype Instability

Neuropathologists have long recognized that the presence of cancer cells with immature morphology (paucity of the cytoplasm, limited cytoplasmic extensions) is associated with higher proliferation indexes and poorer clinical outcomes, and hence higher tumor aggressiveness (Huse et al. 2011).

It is also the case for glial tumors, or gliomas, that are primitive brain tumors affecting adults like children. They are highly heterogeneous and the prognosis of their high-grade forms remains dismal despite the development of treatments funded on DNA alkylation and neo-angiogenesis blockade. Differing clinical evolutions of patients bearing gliomas with similar histological features further complicate the development of curative treatments.

10.4.1 NSC and NPC as the Cell of Origin of Gliomas

The presence of poorly differentiated cells in gliomas and the coexistence within the same tumor of cells of varying phenotypes are compatible with the idea that NSC/NPC could be the cellular sources of these tumors. The frequent development of gliomas in the vicinity of the sub-ventricular zone, a reservoir of NSC with high migratory capacity (Sanai et al. 2005) also favors this hypothesis.

The recent efforts in the characterization of genomic anomalies found in large arrays of gliomas have led to the identification of core mutated pathways in glioma neurosurgical samples, although the mutated components of each pathway vary from one given subtype of glioma to another (for review, see Ohgaki and Kleihues 2009; Vitucci et al. 2011). Interestingly, several of these core pathways are known to be involved in the biology of normal NSC and NPC, such as the EGFR- and PDGFR-driven transduction pathways that are also involved in the maintenance of NSC/NPC properties (Aguirre et al. 2010; Lindberg and Holland 2012; Nicolis 2007). Comparison of the transcriptome profiles of ependymoma, glial tumors that can develop throughout the neuraxis, or pediatric low-grade optic gliomas has provided further arguments for an origin of these tumors in radial glia progenitors (Johnson et al. 2010; Taylor et al. 2005), or NSC from the third ventricle (da Lee et al. 2012; Sharma et al. 2007), respectively.

10.4.2 Glioma Stem Cells

Cancer stem cells (CSCs) are another example of the similarities that can be shared between glioma cells and immature neural cells. The recent efforts in cell biology research on gliomas have indeed shown that proliferation is far from being the only characteristic of immature neural cells borrowed by cancer cells. A significant outcome of these efforts has been the isolation from high-grade gliomas of cancer cells endowed with a mixture of stemlike and tumor-initiating properties (Galli et al. 2004; Hemmati et al. 2003; Ignatova et al. 2002; Singh et al. 2004). These “glioma stem cells” (GSC) grow, like NSC, under the form of self-renewing cellular spheres in serum-free medium supplemented with EGF and bFGF. Remarkably, their

molecular signature combines markers of neural (Sox2, olig2, nestin) and/or embryonic stem cells (CD15/SSEA4, Nanog, Bmi1) and markers of mesenchymal cells (CHI3L1, NAIL, SLUG, TWIST). However, no reliable extracellular marker of GSC has been identified, and their isolation relies now on prospective procedures (Chen et al. 2012; Siebzehnrubl et al. 2011). Beyond this peculiar combination of functional in vitro properties and molecular markers, GSC have the unique ability of conserving the original genomic profile of the tumor from which they are derived and to initiate upon their graft in immunodeficient mice a tumor with morphological and molecular characteristics close to the ones of the tumor of the patient. They have also been found to contribute to endothelial cells composing the neo-vessels that intermingle with the cancer cells (Ricci-Vitiani et al. 2010; Soda et al. 2011; Thirant et al. 2013; Wang et al. 2010). On the contrary, glioma cells grown in serum require several passages prior to being able to initiate a tumor in vivo, with characteristics differing from the original patient's tumor (Lee et al. 2006). In addition, GSCs respond to short-term exposure to serum with downregulation of their stem-like markers and loss of their tumor-initiating property. A growing body of evidence supports that these self-renewing tumor cells determine a tumor's behavior, including proliferation, progression, invasion, and—most importantly—a great part of resistance to therapies (Bao et al. 2006; Bleau et al. 2009; Murat et al. 2008; Thirant et al. 2011). Whether or not these glioma cells with stemlike properties correspond to the progeny of NSCs or NPCs that were the initial targets of the oncogenic hits remains an open question. The striking similarities between the molecular pathways responsible for the maintenance of NSCs with those responsible for the maintenance of GSCs favor the first possibility. The EGFR/PI3K/AKT/mTOR and the Shh/gli signaling networks are two prominent examples of these pathways (Bar et al. 2007; Clement et al. 2007; Lai et al. 2003; Mazzoleni and Galli 2012; Shi et al. 2008; Xu et al. 2008). It should, however, be noted that GSC self-renewal relies on Nanog (Fareh et al. 2012; Li et al. 2011; Mathieu et al. 2011; Po et al. 2010; Turchi et al. 2013; Zbinden et al. 2010) that is not expressed in the normal human brain (Guo et al. 2011). Nanog is best known as a component of the canonical transcription factor network that characterizes embryonic stem cells (nanog-sox2-Oct4) (Boyer et al. 2005). On the other hand, prospective isolation of GSCs has not been performed with success in all tumors of a given category in children as in adults. Although technical problems (e.g., quality of the surgical sample, culture cocktail, mode of cell isolation) cannot be ruled out, the positive association of increased frequency of GSC detection with enhanced tumor grade (Ignatova et al. 2002; Panosyan et al. 2010; Patru et al. 2010; Thirant et al. 2011) suggests that GSCs are not present at all stages of tumor development. The possibility that glioma cells acquire properties of stem cells under adequate environmental pressure cannot be ruled out, but remains to be demonstrated. Finding a set of gene enrichment corresponding to an embryonic stem cell signature in glioblastoma as compared to lower-grade glioma favors this possibility (Ben-Porath et al. 2008).

10.4.3 Acquisition of Immature Features by Glial Cells in the Injured Neural Tissues

The ability of normal glial cells to regress to an earlier stage of their development has been documented in vitro upon enhanced mobilization of growth factor signaling pathways, which have been repeatedly dysregulated in cancer cells. Oligodendrocyte progenitors can revert into multipotent stem cells in response to bFGF exposure (Kondo and Raff 2000). PDGF overexpression achieved with viral transduction of cultured astrocytes induces acquisition of the morphology and molecular markers of glial progenitors (Dai et al. 2001). A simple manipulation of the astrocyte environment can also alter the state of differentiation of astrocytes. Lengthened exposure of differentiated mouse cortical astrocytes to TGFα, acting via EGFR, results in a two-step progressive and functional conversion of the cells, first within 7 days into progenitor-like cells capable of giving birth to cells of the neuronal lineage and second within weeks into neural stemlike cells (Sharif et al. 2007). TGFα first induces astrocytes to progressively change from a polygonal shape to a bipolar form similar to radial glial cells, then to express the molecular markers of this progenitor population, and finally to exhibit their functional properties. They supported the migration of embryonic neurons along their processes and gave birth to cells expressing neuronal markers and possessing the electrophysiological properties of neuroblasts. Lengthening astrocyte exposure to TGFα to 10 months resulted in the appearance of floating cellular spheres that were self-renewing, could be clonally derived from single cells, and differentiated into cells of the neuronal and astrocyte lineages (Sharif et al. 2007). These results suggest the existence of an astrocyte population capable of regressing progressively into immature states and demonstrating a remarkable potential of astrocytes to undergo functional plastic changes in response to a single change in their environment.

As previously mentioned, upregulation of EGFR and its ligands accompanies astrogliosis developing in response to brain injury and can be sufficient to trigger astrogliosis throughout the neural axis (Birecree et al. 1988; Ferrer et al. 1996; Jin et al. 2002; Junier et al. 1993; Lisovoski et al. 1997; Liu and Neufeld 2004; Nieto-Sampedro et al. 1988; Planas et al. 1998; Rabchevsky et al. 1998). In line with these observations, Buffo and colleagues have shown that reactive astrocytes can dedifferentiate towards a more immature state in vivo and exhibit stemlike cell properties when transferred in vitro (Buffo et al. 2008). Forced expression of neurogenic transcription factors can reprogram into neurons, early postnatal astrocytes, or reactive astrocytes derived from an injured adult cortex (Heinrich et al. 2010). Of note, the stemlike potential of reactive astrocytes appears to be restricted to cells that have reentered the cell cycle (Sirko et al. 2013), illustrating the link between cell proliferation and state of differentiation. The reparative potential of astrocytes is further suggested by the recent finding that astrocytes in the striatum of adult mice can be converted into neurons following cell-specific lentiviral delivery of a set of neuronal reprogramming factors (achaete–scute complex-like 1/Ascl1, brain-2/Brn2a), myelin transcription factor-like 1/Myt1l) (Torper et al. 2013).

10.4.4 Permissiveness of Dedifferentiated Astrocytes to Cancerous Transformation

This permissiveness of astrocytes to reprogramming makes them particularly attractive targets for developing brain repair therapies, although the consequences of diverting reactive astrocytes from their normal role have to be first carefully evaluated. Suppression of reactive astrocytes reentering the cell cycle in response to spinal cord injury (stab wound or crush) has, for example, been shown to worsen the severity of the injury, resulting in widespread demyelination, failure to restore the blood–brain barrier, and subsequent enhanced neurodegeneration (Faulkner et al. 2004). Another risk is to promote astrocyte susceptibility to cancerous transformation, as already put forth by Silver and Steindler (Silver and Steindler 2009). Several of the genomic alterations prevalent in human gliomas, although insufficient by themselves to promote cancerous transformation, have been shown to induce a dedifferentiation of astrocytes (Bachoo et al. 2002; Dai et al. 2001; Fraser et al. 2004; Sharif et al. 2007). Two studies have examined whether this altered state of differentiation of astrocytes might sensitize the cells to subsequent oncogenic events. One has shown that introduction of a mutated, constitutively active form of EGFR in cultured *INK4a/ARF* −/− astrocytes, resulted in their transformation in tumor cells capable of forming high-grade gliomas when transplanted into the brain (Bachoo et al. 2002). The other examined the sensitivity to cancerous transformation of astrocytes converted into progenitor-like cells in response to prolonged TGFα treatment. The cells had a normal genomic profile and did not form tumors when grafted into the brain of nude mice (Fig. 10.3). In contrast, when astrocytes dedifferentiated with TGFα were submitted to oncogenic stress using gamma irradiation, they acquired cancerous properties, forming high-grade glioma-like tumors after brain grafting. On the other hand, gamma irradiation was without effect on astrocytes that were not treated with TGFα (Dufour et al. 2009). Newborn mice have been used to achieve somatic delivery of various genomic alterations found in human gliomas, either in immature cells expressing nestin or in cells considered astrocytes because of GFAP expression. For this purpose RCAS-TVA (replication-competent avian sarcoma-leukosis virus splice acceptor-avian receptor tv-a) vector has been largely used (von Werder et al. 2012). RCAS viruses bind to dividing cells. Consequently, their capacity to infect cells is inversely correlated to the age of the animal (Huse and Holland 2009). Most of the data accumulated with this experimental model indicate a higher probability of glioma development when the mutations are introduced into immature cells under the control of the nestin promoter (Shih and Holland 2004). However, the young age of the mouse, coupled with the well-known expression of GFAP in radial glial cells (Alves et al. 2002) and in NSC (Lim and Alvarez-Buylla 1999) does not definitively determine that the GFAP-expressing cells were astrocytes. Of note, introduction of a single genetic alteration in mouse models has proven to be insufficient to induce glioma formation in the vast majority of the cases. Overall, the transfer of genetic alterations found in human glioma into mouse models has shown that gliomagenesis depends on a

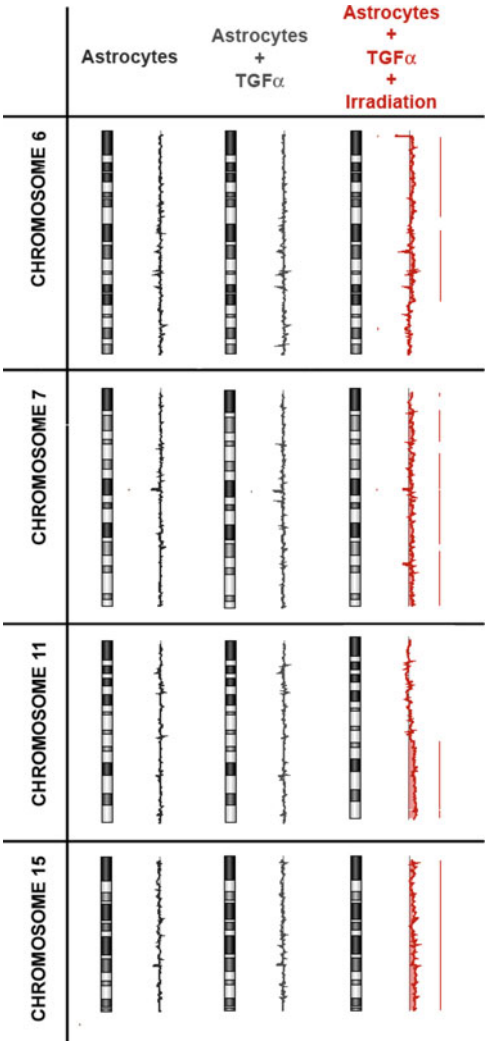

Fig. 10.3 Genomic profiling of dedifferentiated astrocytes. Example of CGH array profiling of astrocytes maintained in defined medium (astrocytes), dedifferentiated into neural progenitor-like cells in response to 7-day exposure to TGFα before (astrocytes + TGFα) and after (astrocytes + TGFα + irradiation) γ-irradiation. Nonirradiated astrocytes had the same genomic profiles, regardless of their exposition to TGFα. Only irradiated TGFα-treated astrocytes exhibited genomic anomalies corresponding noteworthy to gains in chromosomes 6, 7, 11, and 15

dysregulation of the cell cycle, associated with an overactivation of the transduction pathways governed by Ras and Akt (Chen et al. 2012; Rankin et al. 2012). For example, loss of the tumor suppressor gene *Ink4a–Arf* that negatively regulates entrance into the cell cycle (Fig. 10.2) does not trigger gliomagenesis. Likewise, a constitutively activated mutated form of *EGFR* does not initiate by itself gliomagenesis, regardless of the promoters driving its expression (i.e., GFAP or Nestin). Introduction of mutations resulting in overactivation of the transduction pathways located downstream of the tyrosine kinase receptors, such as a constitutively activated form of *Ras*, is also incapable of triggering cancerous transformation of neural cells. On the other hand, combination of mutant EGFR or mutant Ras expression with *Ink4a–Arf* loss results in glioma development (Holland et al. 1998; Uhrbom et al. 2002). One exception is the overexpression of PDGF-B, sufficient by itself to trigger the formation of glioma (Dai et al. 2001). It remains, however, possible that PDGF-B overexpression induces genomic instabilities favoring the appearance of mutations in genes regulating the cell cycle.

Only few works have tackled glioma modeling by developing mouse models that direct mutations in a cell-specific manner in the adult animal. Delivery of adenoviruses allowing cre-mediated inactivation of the tumor suppressor genes *Nf1*, *p53,* and/or *Pten* inactivation in the sub-ventricular zone at both early postnatal and adult ages induced glioma formation. Gliomas occurred, however, rarely in this experimental setting when adenoviruses were delivered into non-neurogenic regions, such as the cortex and the striatum (Alcantara Llaguno et al. 2009). More recently, *GFAP-CreER* mice and inducible targeting with tamoxifen have been used to question the ability of mature astrocytes localized outside the proliferative niches of the adult brain to be cell sources of gliomas. Glioma modeling was achieved through loss of the tumor suppressors *Pten*, *p53*, and *Rb*. In this model, 50 % of mature astrocytes are targeted for transformation as compared to less than 1 % of the neural precursor cells (Chow et al. 2008). The simultaneous inactivation of *PTEN* and *TP53* generated high-grade gliomas in 87 % of mice (Chow et al. 2011). Twenty-two percent of the tumors were located outside the proliferative niches, indicating that mature astrocytes can also behave as the cell of origin of glioma. Although the lack of specific astrocyte markers still constitutes a limitation of these studies, the results obtained suggest again that astrocytes are less permissive than NSC/NPC to cancerous transformation. The question of a prior dedifferentiation of astrocytes was not addressed in this study, which focused on fully developed tumors. This question was addressed by another group using stereotaxic injections of Cre-inducible lentiviral vectors (containing either shNF1-shp53 or H-RasV12-shp53) in GFAP-cre and synapsin-Cre adult transgenic mice (Friedmann-Morvinski et al. 2012). They established glioma in either case suggesting that even neurons can be subjected to cancerous transformation. Most interestingly, they followed the course over time of glioma development and provide evidence for a dedifferentiation of the targeted GFAP-expressing cells in response to loss of p53 combined with Ras activation. The transduced mature astrocytes were observed to progressively lose GFAP expression while increasing l Nestin and Sox2 expression. Moreover, they identified an increase in the expression of Nanog, a transcription factor indispensable for

maintaining the self-renewal properties of ESC as well as of GSC (see Sect. 10.4.2). Of note, the tumors derived from these astrocytes exhibited a molecular profile akin to the mesenchymal subtype of human glioblastoma, the most aggressive form of gliomas (Friedmann-Morvinski et al. 2012). These data further add to the repeated observations that glioma cells, and especially GSCs, reactivate the expression of factors essential for the reprogramming of differentiated cells into pluripotent cells (Wu and Hochedlinger 2011). This study provides for the first time in vivo results supporting the idea that cancerous transformation of astrocytes implies a transition of the differentiated mature cell towards an immature state akin to NPC/NSC.

In summary, the results derived from the different experimental mouse models of glioma have shown that among the neural cells sensitive to cancerous mutations, those endowed with immature features either innate (NPC/NSC) or acquired (astrocytes) are the most prone to cancerous transformation. These data underscore the need for a thorough examination of the possible consequences of any regenerative strategy on the genomic status of the targeted cells.

10.5 Conclusions

Taken as a whole, these data suggest that gliomas result not only from alterations in the regulation of the cell cycle and of cell survival but also from alterations in the state of differentiation of mature cells, as has been proposed for some epithelial cancers (Harris 2004). As a correlate, all neural cells with stemlike potency are prime candidates for cancerous transformation. A common feature of these cells could be their chromatin architecture, which is at the core of gene patterning and hence cell identity. Chromatin architecture is determined by DNA methylation and posttranslational histone modifications, which result in chromatin states either permissive (euchromatin) or repressive (heterochromatin) for transcription. Chromatin profiling of specific candidate loci performed on a genome-wide scale have shown, for example, that high levels of histone H3 lysine 4 methylation (H3K4me) are associated with promoter and enhancer regions of active or potentially active genes, whereas elevated levels of histone H3 lysine 9 and 27 methylation (H3K9me and H3K27me) are associated with repressed chromatin domains and gene silencing (Barski et al. 2007). DNA methylation, usually associated with gene silencing, is another mode of control of the architecture of chromatin (Coskun et al. 2012). The importance of the chromatin state is best illustrated with the changes in histone methylation marks occurring in ESC along their differentiation into somatic stem cells, somatic progenitor cells, and finally somatic cells. This differentiation is accompanied with alleviation of the repressive methylation marks on pro-differentiating genes and conversely, the acquisition of repressive marks on genes essential for stem cell maintenance (Bernstein et al. 2006; Xie et al. 2013). Studies of histone modifications in ESC have notably revealed novel epigenetic features, which are thought to contribute to the maintenance of pluripotency and cell lineage determination of these cells. Notably, transcriptionally poised lineage-specific

genes are simultaneously marked by opposing H3K4me3 and H3K27me3 modifications (Azuara et al. 2006; Bernstein et al. 2006; Mikkelsen et al. 2007). This "bivalent" chromatin structure is generally resolved as the ESC differentiates with loss of the repressive mark and consequently enhanced transcription of genes controlling cell differentiation and inhibition of cell cycle (Mikkelsen et al. 2007; Xie et al. 2013). Silencing of the genes bearing bivalent marks in ESC appear to be reinforced in carcinoma cells, with recruitment of DNA methylases and subsequent DNA hypermethylation, whereas gene loci with active marks appears protected from DNA methylation (Ohm and Baylin 2007; Ooi et al. 2007; Tsai and Baylin 2011). Future comparisons of chromatin status between the different neural cells found to be susceptible to behave as cell of origin of glioma should tell us whether a stemlike chromatin status is a determinant for cancerous transformation.

Acknowledgements The research of the authors is supported by the Institut National de la Santé et de la Recherche Médicale Inserm (France), INCa (Grant PLBIO2012), and the foundations ARC and La Ligue contre le cancer (Grant Equipe Ligue 2013). E. El-Habr benefited from a fellowship of Région Ile de France.

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About the Editors

Steven G. Kernie is an Associate Professor of Pediatrics and Pathology & Cell Biology at Columbia University in New York and Chief of Critical Care Medicine at Morgan Stanley Children's Hospital at Columbia University Medical Center. His laboratory is interested in how the brain repairs itself following injury. The presence of adult neural stem and progenitor cells in the mammalian brain has awakened new interest and optimism in potential treatment for a variety of acquired brain disorders. The Kernie lab is investigating how adult neural stem and progenitor cells participate in injury-induced remodeling and in identifying genes and drugs that might be important in augmenting their contribution. In order to do this, they have generated a variety of transgenic mice that allow for temporally controlled alterations in the endogenous stem cell population in order to optimize the post-injury remodeling that occurs.

Dr. Marie-Pierre Junier is Research Director at Inserm and co-PI of team Glial Plasticity for the Center of Research Neuroscience Paris Seine at the University Pierre et Marie Curie. Her team showed the permissiveness of astrocytes to re-programming into immature states akin to neural progenitors or neural stem cells. It further demonstrated that these plastic capabilities of astrocytes sensitize them to cancerous transformation. The team is now using cancer stem cells isolated from human adult and pediatric gliomas to understand their differences from normal human neural stem cells. Combining proteomic, metabolic and epigenetic approaches, the team aims at developing new therapeutic strategies against these devastating cancers.

M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3, © Springer Science+Business Media New York 2014

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M.-P. Junier and S.G. Kernie (eds.), *Endogenous Stem Cell-Based Brain Remodeling in Mammals*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4899-7399-3, © Springer Science+Business Media New York 2014